Total flavonoid contents in bamboo diets and reproductive hormones in captive pandas: exploring the potential effects on the female giant panda (*Ailuropoda melanoleuca*)

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Phytoestrogens have been shown to affect the reproductive hormone levels in both humans and animals. As the main category of phytoestrogens, total flavonoids have a particularly important impact on female animals. To investigate the potential relationship between the total flavonoids in bamboo and the reproductive hormones in female giant pandas, urinary samples and dietary bamboo samples were collected from three main breeding locations (Beijing, Shaanxi and Sichuan). The chemical constituents of the total flavonoids in the bamboo were analysed and quantified using high-performance liquid chromatography coupled with a diode array detector (HPLC–DAD). Estradiol (E₂), progesterin (P), testosterone (T), luteinizing hormone (LH), follicle-stimulating hormone (FSH) and prolactin (PRL) were measured via radioimmunoassay (RIA). The results revealed that the total flavonoids in the bamboo from Sichuan were significantly higher than those in the bamboo from Beijing and Shaanxi, and the concentration in bamboo from Shaanxi was higher than that from Beijing (*P* < 0.05). The urinary E₂, P, T, FSH and LH levels in pandas from Beijing were significantly lower than those in pandas from Sichuan and Shaanxi (*P* < 0.05). The concentrations of six reproductive hormones were positively associated with the total flavonoid contents in bamboo. In addition, the birth rate of pandas in Sichuan was significantly higher than the birth rate of pandas in Beijing and Shaanxi (*P* < 0.05). Thus, the flavonoids of bamboo may be related to reproduction and giant pandas might retain a sensitive adaptation to phytoestrogens from bamboo. The total flavonoids of bamboo may play a distinct role in the reproductive success of giant pandas.

**Key words:** Diet bamboo, giant panda, phytoestrogen, reproductive hormone, total flavonoids

**Editor:** Dr. Steven Cooke

Received 8 September 2018; Revised 29 October 2018; Editorial Decision 11 November 2018; accepted 25 March 2019

**Cite as:** Liu H, Zhang C, Liu Y, Duan H (2019) Total flavonoid contents in bamboo diets and reproductive hormones in captive pandas: exploring the potential effects on the female giant panda (*Ailuropoda melanoleuca*). Conserv Physiol 7(1): coy068; doi:10.1093/conphys/coy068.

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Introduction

Phytoestrogens are plant-derived oestrogens that are not generated within the endocrine system but are consumed only by eating phytoestrogenic plants (Bennett et al., 1946; Dusza et al., 2006). Hundreds of plants have been found to contain phytoestrogens, including some well-known isoflavonoids, stilbenes, lignans and coumestans, and these compounds can bind to oestrogen receptors and mimic the action of oestrogens on target organs and cause oestrogenic effects on animals and humans (Dusza et al., 2006; Mlynarczuk et al., 2011). Phytoestrogens are not considered nutrients because they do not participate in any essential biological function (Chen, 2004). However, phytoestrogens have been found to promote growth, improve immunity and lactation performance and even affect the egg production performance of animals (Wang et al., 2013).

Many specific studies have demonstrated the various impacts of phytoestrogens on reproduction in birds and mammals, and these studies showed that phytoestrogens act variably when they are consumed by different species and genders and administered through different routes in captive animals (Murnkies et al., 1998; Setchell and Cassidy, 1999; Ball et al., 2010; Lu et al., 2010; Wasserman et al., 2012a,b; WocBawek-Potocka et al., 2013; Sittipon et al., 2014). Still, the effect of food supplementation in long-term on populations remains unknown and few are published on the research of wildlife (Birnie-Gauvin et al., 2017). Since bamboo accounts for 99% of the diet of giant pandas (Ailuropoda melanoleuca) (Schaller et al., 1985), phytoestrogens may have a substantial influence. However, the impact of total flavonoids in zoo-housed giant pandas has not been reported. To obtain a better understanding of the reproductive process of these animals, the potential relationship between total flavonoids in bamboo diets and the reproductive hormones of giant pandas must be investigated.

Giant pandas feed on a wide variety of bamboo within various localities in China (Zhou and Huang, 2005; Fu et al., 2008). In the Qinling Mountain area of Shaanxi Province, 17 species of bamboo are consumed by giant pandas, including Fargesia denudata, Fargesia rufa and Fargesia dracocephala (Tian, 1989; Huang, 1995; Li et al., 2003; Mo et al., 2004). In Sichuan Province, giant pandas prefer to feed on eight species, including Yushania lineolata, Bashania fangina, and Bashania spanostachya (Fu et al., 1988; Qin et al., 1993; Zhou et al., 1996). Several studies have indicated that different bamboos contain different flavonoid constituents, such as orientin, isoorientin, isovitexin, vitexin and tricin (Wei et al., 2013; Guo et al., 2014; Pan et al., 2014). Even if the same flavonoids are present, the concentration levels will vary significantly among different species of bamboo. For example, the maximum level of flavone C-glycosides was found in Phyllostachys nigra, while the minimum level was found in Phyllostachys propinqua (Li et al., 2004). Additionally, the diversity of flavonon content has been shown to vary based on the geographic distribution of bamboo; the flavonoid level from the Meishan area was the highest and the level in the Yaan area was comparatively lower (Yu et al., 2008).

Giant pandas housed in Chinese zoos are traditionally fed different bamboo species. There are three main captive populations of giant pandas. Giant pandas from Shaanxi Province are primarily fed bamboo from the Phyllostachys genus, whereas those from Sichuan Province are fed Pleioblastus amarus and Phyllostachys bissetii, and those from Beijing are fed P. propinqua. Sichuan and Shaanxi have a significant history of suitable reproductive performance, whereas Beijing presents lower reproductive performance based on a scientific comparison (Xie, 2014).

Female giant pandas from Beijing were moved to Sichuan for breeding since 2011, and ten cubs have been born in Sichuan during 2012 to 2018. Similar husbandry and management were implemented at these three institutes; thus, the question remains as to whether the bamboo diets of these pandas played an important role in their reproductive success. Giant pandas usually have a delayed implantation during the reproduction cycle, so the reproductive hormone are basically at a very low level during the oestrus cycle from March to May (Lindburg et al., 2001; Zhang and Wang, 2003; Zhang et al., 2009). Further, the urine oestriol (E2) level closely correlates to the estrus of giant pandas (Zeng et al., 1994, McGeehan et al., 2002). The objective of this study is to determine whether differences are observed in reproductive hormones levels based on the consumption of different bamboos and to investigate the potential influence of bamboo phytoestrogens on the reproduction of giant pandas.

Phytoestrogens may vary according to bamboo species and distribution (Li et al., 2004; Yu et al., 2008), and their contents may affect the hormone levels of giant pandas. We examined the concentrations of total flavonoids of dietary bamboo from three collections as well as the urinary hormone levels of E2, progesterone (P), testosterone (T), follicle-stimulating hormone (FSH), luteinizing hormone (LH) and prolactin (PRL) among giant pandas in three locations. Specifically, this study aims to determine whether the total flavonoids in different species of bamboos are potentially related to urinary hormone levels in adult female giant pandas.

Material and methods

Animal and bamboo diets

The animals in this study included 11 female giant pandas at three sampling locations (Table 1), and the samples were collected during the reproductive season (from March to May). At the same period of time, urinary samples from the giant pandas and their bamboo diets were simultaneously collected.
by random sampling. Three pandas in this sampling were from Beijing Zoo, four were from the Sichuan Wolong Nature Reserve, and four were from the Shaanxi Rare Wild Animals Rescue and Breeding Research Centre. The main species of bamboo fed to the giant pandas were as follows: *Phyllostachys propinqua McClure* from Beijing, *Pleidolus amurus* from Sichuan and *Phyllostachys aureosulcata Spectabilis* from Shaanxi. Approximately 2–3 kg bamboos for each species were randomly collected from three locations and stored at the low temperature. In accordance with the technical regulations of husbandry and management of the giant pandas published by China standard press (2012), the daily diets in three locations consisted of 75–90% fresh bamboo and 10–25% supplemental feed, which included steamed bread, apples and carrots.

Sample collection
The giant pandas usually eat not only the culms but also the leaves of bamboo from March to May. Fresh whole bamboo diets were collected by random sampling at the three zoos from the same time period of 2014. *Phyllostachys propinqua McClure* from Beijing, *Pleidolus amurus* from Sichuan and *Phyllostachys aureosulcata Spectabilis* from Shaanxi were the main categories of food. All collected bamboos from the three locations were shipped to a Beijing laboratory and stored at 4 °C until further analysis.

| Giant pandas | Birth year | Collections | Urinary sample | Bamboos | Breeding time (years) | Bamboo samples |
|--------------|------------|-------------|----------------|---------|-----------------------|----------------|
| Yinghua      | 2003       | Beijing     | 10             | *Phyllostachys propinqua McClure* | 5               | 20 pieces         |
| Mengmeng     | 2006       | Beijing     | 10             | *Phyllostachys propinqua McClure* | 3               | 3               |
| Jini         | 1993       | Beijing     | 10             | *Phyllostachys propinqua McClure* | 10              | 10              |
| Haizi        | 1994       | Sichuan     | 12             | *Pleidolus amurus* | 8               | 20 pieces         |
| Long Xin     | 2000       | Sichuan     | 12             | *Pleidolus amurus* | 6               | 6               |
| Zizhu        | 2002       | Sichuan     | 12             | *Pleidolus amurus* | 4               | 4               |
| Guo guo      | 1998       | Sichuan     | 12             | *Pleidolus amurus* | 7               | 7               |
| Xin xin      | 2005       | Shaanxi     | 11             | *Phyllostachys aureosulcata Spectabilis* | 3               | 20 pieces         |
| Yang yang    | 2003       | Shaanxi     | 11             | *Phyllostachys aureosulcata Spectabilis* | 5               | 5               |
| Zhuzhu       | 2000       | Shaanxi     | 11             | *Phyllostachys aureosulcata Spectabilis* | 5               | 5               |
| Niuniu       | 1997       | Shaanxi     | 11             | *Phyllostachys aureosulcata Spectabilis* | 7               | 7               |

Determination of the total flavonoid contents from the bamboo diets
The total flavonoids of these bamboo diets were determined and quantified using a high-performance liquid chromatography coupled with a diode array detector (HPLC-UV/DAD) system (Waters Co. Ltd, USA). Whole bamboo samples were dried in an oven at 40 ± 2 °C for 48 h and triturated in an RRH-A400 pulverizer (Zhejiang Province, China). The samples were ground at 800 r/min for 6 min to obtain a fine powder. All samples were then bagged and stored in a desiccator until further analysis.

The analytical standard rutin (quercetin-3-O-rutinoside, 98% purity) was purchased from Sigma-Aldrich (China). Analytical grade ethyl alcohol, sodium carbonate, sodium bicarbonate, and phosphoric acid were purchased from the Beijing Chemical Reagent Company (Beijing, China). Methanol and acetonitrile (HPLC grade) were provided by Dikma Co., Ltd (Beijing, China).

Fine bamboo powder (2.0 g) was immersed in 80 ml 75% (v/v) aqueous ethanol for 24 h and decoloured by adding ~2 g active carbon over one hour. After centrifuging for 5 min, the supernatant was concentrated using rotary evaporation to remove the ethanol. Finally, Millipore-filtered water was added to each sample tube to yield a final volume of 20 ml for analysis. A HPLC-UV/DAD system (Waters, USA) coupled with a C18 column 4.6 mm × 250 mm (Agilent, USA), was used to determine the total flavonoids. Mobile phase A was set by acetonitrile, and the composition of mobile phase B was set by phosphoric acid buffer (pH = 2.8) in water. The isocratic elution using A/B (45:55, v/v)
was applied at a total flow rate of 1.0 ml/min. The absorbance of the signal using a diode array detector was 355 nm.

Radioimmunoassay determination of urinary hormones

The urinary hormones E$_2$, P, T, FSH, LH and PRL were obtained from the three giant panda locations (Beijing, Shaanxi and Sichuan) and analysed using E$_2$, P, T, FSH, LH and PRL radioimmunoassay (RIA) kits from Beijing North Institute of Biological Technology (Beijing, China). The urinary hormone concentrations were measured by a 125 I-based RIA. The urine samples were corrected by the creatinine value to control the variability in the urine concentration. The units of hormonal values were transformed to mass units per milligrams of creatinine (Taussky, 1954). These assays were performed base on the manufacturers’ recommendations for urine samples. All samples were analysed simultaneously using the same microtiter plate to decrease unexpected variations (Cekan, 1975). Recovery of known amounts of the six hormones respectively added to a pool of diluted panda urine (50 μl, 1:5) ranged 78–103% ($n = 6$), which met the test requirement. The liners of six hormones added in diluted urine samples showed a good relationship ($r = 0.98–0.99$). The intra-assay and inter-assay coefficients of variations were <10 and 15%, respectively. Hormonal values under 0.1 mg ml$^{-1}$ were considered below the range of sensitivity and replaced by the limit of detection. Undetected values accounted for <5% of the total measurements.

Statistical analysis

The resulting hormone values were transformed into mass units per milligrams. The equation for calibration transformations is 1 μmol L$^{-1} = (8840$ mg ml$^{-1})^{-1}$. The annual birth rate was determined by the number of newborns among the total number of animals in a breeding area in a year. The average annual birth rates of three locations were calculated according to the studbook from 2002 to 2014 (Xie, 2014).

The Kolmogorov–Smirnov test was applied to determine whether the dataset matched the assumption of normality. After its application, the proper statistical tests had been chosen on the different datasets. The Kruskal–Wallis H test as nonparametric test was used to identify the hormone differences among the three captive groups. The Mann–Whitney test was used to compare the differences in bamboo flavonoids between two collections. Spearman tests were applied to determine the significance of the correlations between the hormone parameters and total bamboo flavonoids. One-way ANOVA as parametric test was used to analyse the birth rate differences in three locations. The homogeneity of variance was analysed using Levene’s test, and multiple comparisons within groups were performed using post hoc tests.

SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses. Differences were considered significant at $P < 0.05$. The data were described as the mean ± SE.

Results

Total flavonoid contents in the bamboo diets

The total flavonoid content in the bamboo diets of pandas from Sichuan was significantly higher than that in the bamboo diets of pandas from Beijing (Mann–Whitney test $Z = -2.121$, $P = 0.034$; Fig. 1). Additionally, the total flavonoid content in the bamboo from Shaanxi was lower than that in the bamboo from Sichuan ($Z = -2.309$, $P = 0.021$; Fig. 1) and statistically higher than that in the bamboo from Beijing ($Z = -2.121$, $P = 0.034$; Fig. 1).

Reproductive hormone levels in pandas at the three locations

The overall urinary E$_2$, P, T, FSH and PRL levels were significantly different among the three locations, whereas statistically significant differences were not observed for LH (Kruskal–Wallis test, Table 2). The urinary E$_2$ in Beijing zoo samples was significantly lower than that in the Shaanxi and Sichuan zoo samples (Mann–Whitney test, Table 2 and Fig. 2a). The urinary E$_2$ from the Sichuan pandas was significantly higher than that from the Shaanxi pandas (Table 2 and Fig. 2a). However, the P and T of the Shaanxi zoo pandas were the highest among the three locations (Table 2 and Fig. 2b and c), whereas the P and T of the Beijing zoo pandas were significantly lower than those of the other panda groups (Table 2; $P < 0.05$, Fig. 2b and c).

Although the levels of LH did not show differences among the three locations (Kruskal–Wallis test, Table 2), the measurements of the Beijing animals differed from those of the Sichuan and Shaanxi animals (Mann–Whitney test, $P <
Table 2 Analysing the six reproductive hormones of giant pandas at three collections

| Item      | Main effect | Group comparison |
|-----------|-------------|------------------|
|           | df | \(X^2\) | \(P\) | Beijing vs. Shaanxi | Beijing vs. Sichuan | Shaanxi vs. Sichuan |
| E2 (ng mg\(^{-1}\)) | 2  | 8.909 | 0.012\(^*\) | + | + | + |
| P (ng mg\(^{-1}\)) | 2  | 8.956 | 0.011\(^*\) | + | + | + |
| T (ng mg\(^{-1}\)) | 2  | 8.227 | 0.016\(^*\) | + | + | + |
| LH (mIU mg\(^{-1}\)) | 2  | 5.053 | 0.080 | – | + | – |
| FSH (mIU mg\(^{-1}\)) | 2  | 7.136 | 0.028\(^*\) | + | + | – |
| PRL (mIU mg\(^{-1}\)) | 2  | 7.053 | 0.029\(^*\) | – | + | + |

Kruskal–Wallis test was used to analyse main effect. Mann–Whitney test was performed between groups. The significant threshold value equals to 0.05. The result interprets: ‘*’ shows the significant value \((P < 0.05)\), while ‘–‘ presents the not significant value \((P > 0.05)\).

Discussion

The composition of total flavonoids in a plant is generally dependent on the species, although it can be altered by environmental factors (Whitten et al., 1995). Even in similar bamboo species, the concentration of total flavonoids varies according to different geographic distributions (Yu et al., 2008). In this research, the concentrations of total flavonoids were different in the three locations. The total flavonoid content in \(P. propinqua\) from Beijing was lower than those in \(P. amarus\) from Sichuan as well as those in \(P. aureosulcata\) in Shaanxi. Our results were consistent with previous studies in which the total flavonoids of \(P. propinqua\) were the lowest among eight bamboo species (Li et al., 2004).

According to the urinary hormone levels, we observed that the hormones (except for PRL) in the Beijing samples were lower than those in the Shaanxi or Sichuan samples. All reproductive hormones in the Sichuan samples were significantly higher \((P < 0.05; \text{Fig. } 2f)\). However, there was no significant difference in PRL samples between the samples from the Shaanxi and Beijing pandas \((P > 0.05; \text{Fig. } 2f)\).

Total flavonoid contents in the bamboo diets and reproductive hormone levels in the pandas

To determine whether the total flavonoids of bamboo diets affected the level of reproductive hormones in female giant pandas, urinary samples and bamboo samples from the three study locations were analysed via Spearman’s correlation. All six reproductive hormones showed a positive correlation with the total flavonoids in the bamboo species. The urinary E2 was statistically correlated with the concentration of total flavonoids \((r = 0.726, P < 0.01; \text{Fig. } 3a)\). Similar to urinary E2, the P \((r = 0.490, P < 0.01; \text{Fig. } 3b)\), T \((r = 0.468, P < 0.01; \text{Fig. } 3c)\), LH \((r = 0.465, P < 0.01; \text{Fig. } 3d)\) and FSH \((r = 0.503, P < 0.01; \text{Fig. } 3e)\) concentrations were significantly correlated with the total bamboo flavonoids, although urinary PRL was less strongly correlated with the total bamboo flavonoids \((r = 0.279, P = 0.03, \text{Fig. } 3f)\).

Birth rates at the three locations

Significant differences were observed among the three locations (one-way ANOVA \(F = 5.004, P = 0.015\)). The average annual birth rate in the Sichuan facility \((20.60 \pm 2.90)\) was significantly higher than that in the Beijing and Shaanxi facilities (post hoc tests; \(P = 0.006\) and \(P = 0.028\), respectively; Fig. 4). The average annual birth rate in the Beijing facility was lower than that in the Shaanxi facility \((6.75 \pm 3.01, 9.89 \pm 3.72\), respectively\), although the difference was not statistically significant \((P = 0.501; \text{Fig. } 4)\).
the reasons for this variation might be seasonal fluctuations in the concentration of flavonoids in different bamboos (Su et al., 2011). Moreover, the total flavonoid contents in various bamboos are higher in spring (Lü et al., 2011; Pan et al., 2014). Zhao et al. (2013) found that giant pandas preferred a slightly bitter bamboo species in which the bitter taste was primarily associated with certain flavonoids. Although the total flavonoids in the bamboo diets were only found in trace amounts, the target compositions could be accumulated in vivo due to daily consumption (Schaller et al., 1985; Sarita et al., 2008). Wynne-Edwards (2001) found that a lower concentration of phytoestrogens compared with endogenous oestrogens was still
related to reproduction in domestic mammalian herbivores. The phytoestrogens of bamboo diets are therefore considered a main factor that influences giant pandas (Yuan et al., 2015), and the concentrations of total flavonoids may have a close relationship with panda reproduction.

E2 is the foundation of oestrus in female mammals, playing an important role of beginning in reproduction (Wallen and Goy, 1977; Lipschitz, 1997). Little is known about the activity of phytoestrogens on reproduction in terrestrial mammals, although in recent years, phytoestrogens and their

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**Figure 3** The relationship between total flavonoids of bamboo and urinary hormones in giant pandas. (a) Estradiol, (b) progesterone, (c) testosterone, (d) LH, (e) FSH, and (f) PRL. The hormone measurements were transformed by logarithm for statistics.
levels of giant pandas in Beijing were relatively low compared with the levels of pandas from Sichuan and Shaanxi, and smaller total flavonoid amounts were found in the bamboo species fed to Beijing pandas. The total flavonoids in various bamboos influence the reproductive hormones in zoo-housed giant pandas. These findings complement relevant studies on dietary phytoestrogens and highlight the potential effects of total flavonoids for increasing the hormone levels of E₂ and P. Based on these findings, an improved dietary plan may be necessary for giant pandas in zoos. Further research is needed to clarify the specific mechanisms underlying the regulation of reproduction by phytoestrogens.

Acknowledgements

The authors thank Mr Jinguo Zhang and Dr Dingzhen Liu (Beijing Normal University) for collecting data. We would also like to thank keepers and lovely giant pandas.

Funding

This study was financially supported by the Giant panda international cooperation fund of State Forestry Bureau (2017) and Beijing Park Management Center project (ZX20170).

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