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The beneficial role of plant secondary compounds in giant panda foraging ecology

Fei Yang[1,2] · Ronald R. Swaisgood[3] · Yuan Liu[1,2] · Tingting Fang[1,2] · Yi Dai[1,2] · Megan A. Owen[3] · Zejun Zhang[4] · Le Wang[1,2] · Shibin Yuan[1,2]

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Abstract

Forage selection by mammalian herbivores has shown to be influenced by plant nutritional content, but the role of plant secondary compounds (PSCs) on forage selection is less well understood. Here, we studied the role of PSCs in giant panda (*Ailuropoda melanoleuca*) foraging strategies; examined seasonal and age class variation in PSC composition in the panda's principal food resource, bamboo (*Bashania fargesii)*; evaluated anti-oxidant and antibacterial effects of bamboo extract; and determined how panda's seasonal movements and foraging patch selection which were determined by GPS collars related to patterns of PSC concentrations in bamboo. Panda's selection of foraging sites indicated positive selection for several PSCs, including flavonoids, alkaloids, and tannins. Pandas primarily ingested bamboo leaves, as opposed other parts of the bamboo, during the time of year when many PSC concentrations were at their highest. Further, pandas prefer to forage on younger bamboo, which contains higher concentrations of alkaloids and antibacterial activity than older bamboo. As might be expected for compounds that can have positive or negative biological effects depending on dose, pandas appeared to select both for and against some PSCs depending on context. Ex situ experiments showed that flavonoids and alkaloids were influential antioxidants and tannins and alkaloids had high levels of antibacterial activity. Panda foraging sites were characterized by high anti-oxidant activity. Variation in PSC content of bamboo on the landscape may have profound effects on pandas, including parasite control, protecting against cancer, improved cardiovascular health, and disease prevention. These potential roles of PSCs should receive greater attention in ecology and conservation.

Keywords Diet · Foraging · Nutritional ecology · Self-medication · Threatened species

Introduction

A primary goal of nutritional ecology in terrestrial herbivores is to improve understanding of the factors influencing foraging decisions and diet selection (Choat and Clements 1998; Raubenheimer et al. 2009). Mammalian herbivores are adapted to the ingestion of plants from which they derive the nutrients and energy for body maintenance, growth, and reproduction. However, a major nutritional challenge is posed by the concomitant presence of plant secondary compounds (PSCs), which are organic compounds usually considered to have no function in the primary metabolic processes of the plant, and which can have negative effects on consumers post-ingestion (Rosenthal and Berenbaum 1992). Recent reviews indicate a primary role of secondary compounds in anti-herbivore defense (Agrawal and Weber 2015). In fact, mammalian herbivores have evolved a variety of mechanisms for avoiding the negative effects of PSCs,

Handling editor: Heiko Georg Rödel.

✉ Le Wang
wangle_0806@163.com

✉ Shibin Yuan
yshibin1020@cwnu.edu.cn

1 Key Laboratory of Southwest China Wildlife Resources Conservation (Ministry of Education), China West Normal University, Nanchong, Sichuan, China

2 Nanchong Key Laboratory of Wildlife Nutrition Ecology and Disease Control, China West Normal University, Nanchong, Sichuan, China

3 San Diego Zoo Global, 15600 San Pasqual Valley Road, Escondido, San Diego, CA 92027-7000, USA

4 Chengdu Normal University, Chengdu, Sichuan, China

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including avoidance, regulation of intake, and biotransformation or detoxification of PSCs to aid in their excretion (Freeland and Janzen 1974; Dearing et al. 2005). Foraging animals may discontinue ingestion of a plant species with PSCs or select different foraging patches or alternative food resources to mediate cumulative exposure to toxins (Iason and Villalba 2006). With most PSCs, some ingestion is tolerated but toxicity becomes problematic at higher doses, overwhelming any physiological mechanisms to counter toxicity (Feng et al. 2009).

Less well understood are the potential positive leverage of some PSCs, including nutritional and non-nutritive effects (Iason 2005; Feng et al. 2009). Although the toxicity of many PSCs has been perceived, mammalian herbivores do not completely avoid PSCs, and there is evidence that they may sample plants that contain PSCs (Iason 2005). At low doses, many PSCs have been associated with beneficial effects on human health (Crozier et al. 2008; Wink 2015). The amounts of PSCs ingested may be governed by the need to balance nutrient acquisition with possible toxicological effects that increase with amount ingested. In fact, some PSCs in small amounts can have beneficial physiological consequences in animal species, including improved immune function, protection from oxidative cell damage, and other possible benefits (Iason 2005; Raubenheimer et al. 2009). However, strong evidence for many of these effects is notably lacking.

The giant panda (*Ailuropoda melanleuca*) makes a good case for the study of foraging adaptations to PSCs. Giant pandas, vulnerable to extinction but beginning to recover (Swaisgood et al. 2016), are highly specialized bamboo consumer, with bamboo comprising > 99% of their diet (Schaller et al. 1985). Theory predicts and meta-analyses support the hypothesis that specialist herbivores have a tighter co-evolutionary linkage between the herbivore and the PSCs present in their primary food source, and thus have a higher tolerance to the presence of PSCs in their diet than do generalists (Cornell and Hawkins 2003). Further, giant panda foraging ecology and digestive efficiency are reasonably well studied, helping to generate hypotheses about the selection of bamboo based on PSC content.

Giant pandas are a member of the order Carnivora and, therefore, do not have many of the adaptations associated with mammalian herbivores, retaining a gastrointestinal tract similar to their more carnivorous ancestors. Yet, bamboo is a very poor source of nutrition, high in fiber and low in energy and other nutrients. These incongruent facts were puzzling for many years, and the main means of proposed compensation were morphological (the famous panda's thumb, modifications to dentition and the muscular apparatus associated with chewing) and behavioral (low activity levels, increased time spent foraging, up to 14 h each day) (Schaller et al. 1985). More recent evidence has also revealed physiological adaptation (low basal metabolic rates) (Nie et al. 2015a), cellulose-digesting microbes in the gut microbiome that aid in the digestion of their high cellulose diet (Zhu et al. 2011), and genes encoding improvements in digestive physiology such as elevated efficiency in the digestion of proteins and vitamins (Hu et al. 2017). Because of their near-complete reliance on a single food source and the increasing evidence for highly specialized digestive abilities, we hypothesized that giant pandas might be able to not only tolerate PSCs, but they may have evolved the capacity to benefit from the consumption of some PSCs.

Fortunately, a great deal is known about panda foraging preferences, and this background information provides opportunities to examine how these preferences map onto PSC content in bamboo. Pandas demonstrate several foraging strategies that may maximize the nutritional value of the bamboo they consume (Schaller et al. 1985; Reid and Hu 1991; Zhang et al. 2009; Nie et al. 2015b; Wei et al. 2015b; Hong et al. 2016; Li et al. 2017). Factors influencing the selection of bamboo by pandas include bamboo age and life stage, part of a plant (leaves versus culm), and characteristics of the location where bamboo is growing, and all of this is subject to seasonal and geographic variation. However, the nutritional basis of these foraging decisions is not well understood (but see Nie et al. 2015b).

Because plants may contain thousands of different PSCs, to evaluate the impacts of PSCs on panda health and reproduction, we focused our study on flavonoids, tannins, and alkaloids. These PSCs are hypothesized to have beneficial effects on health and reproduction. Wild pandas are known to have high parasite loads (Zhang et al. 2008, 2015) and viral and bacterial disease prevalence can be high in captive animals (Mainka et al. 1994; Qin et al. 2010; Feng et al. 2016), though less is known about disease in wild pandas. Thus, parasites and diseases are considered emerging issues that may threaten wild panda populations (Swaisgood et al. 2016). Because plants contain thousands of different PSCs, it is necessary to target specific PSCs with known or hypothesized beneficial effects on health and reproduction. Among these, we nominated flavonoids, tannins, and alkaloids. Flavonoids can have anti-oxidative, anti-mutagenic, anti-inflammatory, and anti-carcinogenic properties in humans, enhancing important biological functions involved in disease prevention among other benefits (Panche et al. 2016). Recent research has also demonstrated seasonal variation in flavonoids in bamboo and that seasonal elevational migration in pandas is correlated with flavonoid content, suggesting a possible dietary preference for flavonoids (Wang et al. 2020). While tannins have many known deleterious effects (Iason 2005), some tannins have been found to serve the same biological functions as found for flavonoids, mostly in herbivorous livestock, and are now recommended in low concentrations because of positive effects on parasite and bacterial load, milk production, reproduction, and other health metrics (Min et al. 2003; Athanasiadou and Kyriazakis 2004; Huang et al. 2018). Indeed some studies have shown animals capable of self-medication, increasing consumption of tannins when parasitized (Lisonbee et al. 2009). Further, while high levels of tannins may compromise protein digestion, low doses may reduce protein degradation during digestion. Alkaloids are generally avoided by herbivores due to their potential toxicity, and are especially known for neurological effects, paralysis and sometimes death (Gemede and Ratta 2014). However, in small doses, alkaloids can have anti-parasitic function (Athanasiadou and Kyriazakis 2004; Villalba and Provenza 2007).

Flavonoids, tannins and alkaloids are known to occur in bamboo which has been widely utilized in the health food and medicinal industry in Asia for over 1000 years (Shibata et al. 1976; Lin et al. 2008; Hong et al. 2016; Wang et al. 2020). Bamboo leaf extract (BLE) has a multitude of health-supporting properties (Mittra et al. 2000; Cottigli et al. 2001; Li et al. 2002; Odontuya et al. 2005; Conforti et al. 2009; Orrego et al. 2009; Ni et al. 2014). It has been hypothesized that the biological function of bamboo leaves is due to the presence of PSCs, such as flavones, phenolics and triterpenoid (Zhang et al. 2007a).

Here we examined the possible role of PSCs in giant panda foraging decisions. Exploratory in nature, our research targeted promising candidate PSCs likely to be found in pandas' diet and we evaluated how panda foraging patterns related to the presence of specific classes of PSCs. We evaluated the degree to which the abundance of PSCs in bamboo varies as a function of site, season, and bamboo age. We hypothesized that preferred foraging sites may contain these PSCs with higher content than randomly selected control sites, reasoning that evidence for selection for specific PSCs is indicative of positive nutritional effects, whereas selection against PSCs is indicative of avoidance and anti-herbivore defense properties. Further, we hypothesized that pandas' known preference for young bamboo leaves over old ones might be related to PSC levels. Thus, we predicted that young versus old bamboo leaf would contain different levels of PSCs, reflecting pandas' foraging preferences. We believe these data will provide new insights into the panda's nutritional ecology, including constraints on optimal foraging based primarily on energy and protein. Further, we believe a more nuanced understanding of the panda's foraging habitat will provide improved information for conservation managers making decisions about protecting, restoring or managing habitat.

Materials and methods

Study area

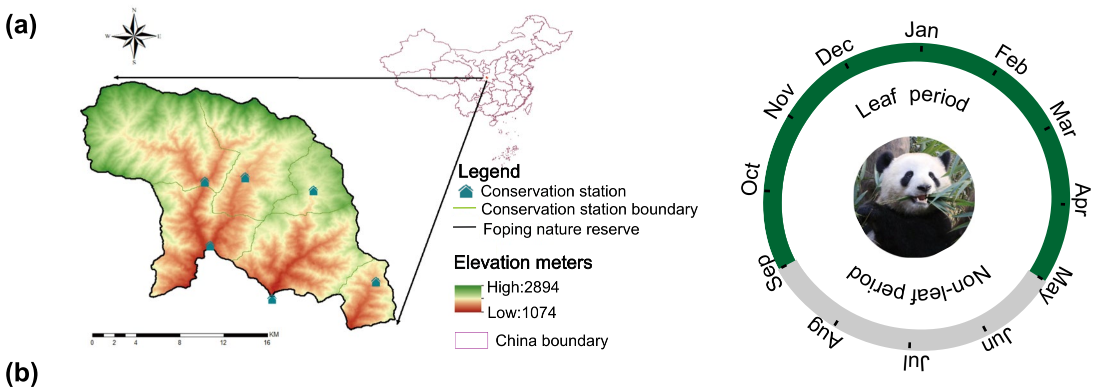
Our study was conducted in Foping National Nature Reserve (hereafter "Foping", Fig. 1a), a 293 km^2 protected area in Shaanxi, China (102°48′–103°00′ E, 30°19′–30°47′ N). The reserve was established in 1978 primarily for the preservation of giant pandas and is located on the southern slopes of the Qinling Mountains. Across the Qinling Mountains, giant pandas live at low elevations from September to June and at high elevations at all other times (Zhang et al. 2007b). Elevations in the reserve range from 980 to 2904 m a.s.l. Foping contains the highest density of wild giant pandas in the world (State Forestry Administration-China 2006). Two bamboo species, *B. fargesii* (usually below 2000 m) and *Fargesia qinlingensis* (usually above 2000 m) are the main food resource for pandas in the reserve (Wei et al. 2015b).

GPS tracking and sample collection

To track movements relating to food resource utilization, we fitted four wild giant pandas with GPS/VHF radio collars (Lotek Wireless Inc, Ontario, Canada) and monitored them from 16 November 2010 to 1 April 2012 (Fig. 1b). GPS collars were programmed to collect location data every 3 h and we used a handheld command unit (Lotek Wireless Inc, Ontario, Canada) to download GPS data when a collared animal was within 100 m (Wei et al. 2015b).

Our study focused on *B. fargesii* leaves, the panda's predominant food resource for much of the year. Because pandas consume primarily leaves during the period from September to April, we categorized this season as the "Leaf period" (Nie et al. 2015b). During the remaining months (May to August), pandas consume *B. fargesii* shoots or migrate to higher elevation to consume *F. qinlingensis*, which we label the "Non-Leaf period," referring to the fact that they do not consume *B. fargesii* leaves at this time (Fig. 1a). GPS data acquired during the Leaf period are presented in Fig. 1b. Used foraging patches were determined by GPS location data and confirmed by signs of foraging activity. Panda foraging sites are easily determined due to the characteristic bamboo "stumps" left behind after foraging and the presence of droppings. Control sites with no feeding trace were established not less than 200 m away from foraging site.

We sampled *B. fargesii* leaves at elevations ranging from 1200 to 1900 m. We followed panda movements using GPS data and sampled feeding and controls sites



(b)

Individual	Sex	Age	Days tracked	Total GPS locations	GPS locations in Leaf period
Xiyue	Male	Adult	483	3278	1248
Cancan	Male	Adult	331	2437	718
Xiaomei	Female	Adult	463	3241	1486
Niuniu	Female	Adult	398	840	1126

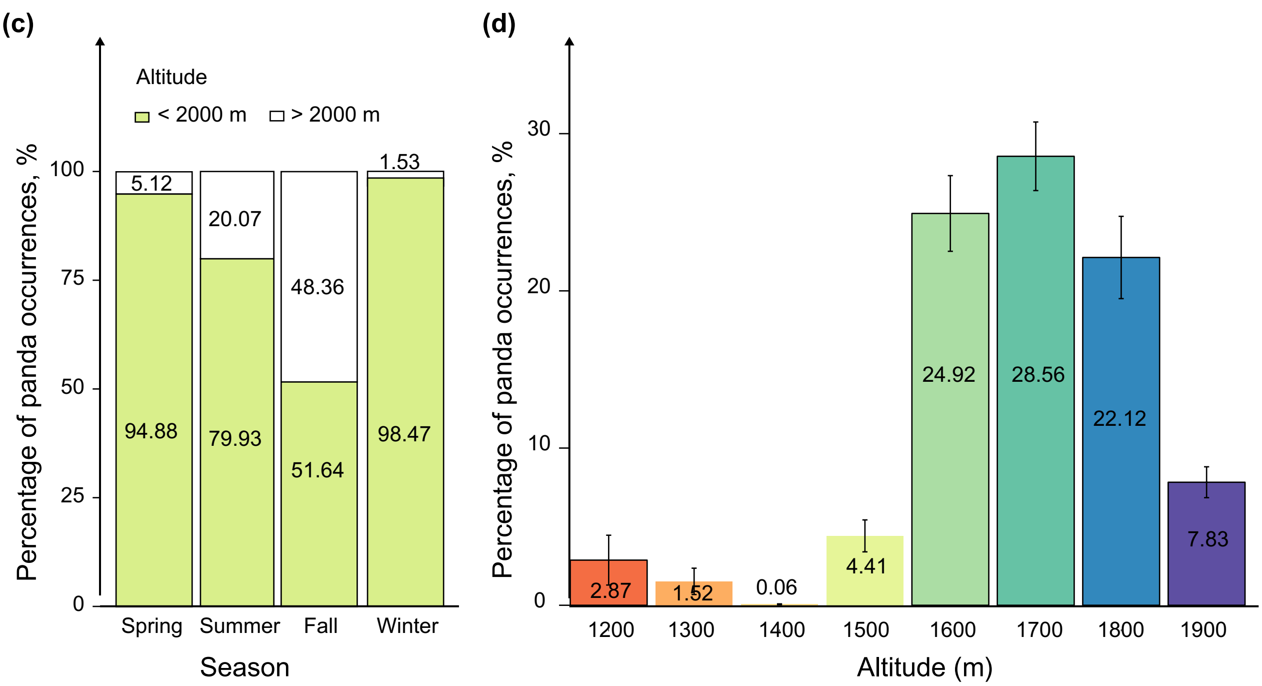


Fig. 1 Study area and altitudinal utilization. **a** Left, Foping National Nature Reserve (FNNR); Right, the seasonal dietary pattern of giant pandas in FNNR. **b** GPS tracking effort of four giant pandas. **c** Proportion of panda GPS locations above and below 2000 m in elevation. **d** Elevational preference of giant pandas during the Leaf period (error bars indicate +/− SD)

within 30 days of visitation by a giant panda, ensuring that our analyses reflected compounds present at the time of panda selection for foraging. At each foraging patch we randomly placed three 1 m^2 plots and collected bamboo leaves for each age group: annual (< 1 year), biennial (1-2 years), and perennial (≥ 3 years). Age of *B. fargesii* was determined as described by (Zhang et al. 2009).

Phytochemical analysis

To obtain BLE, the leaves underwent a series of procedures. Firstly, they were washed and drained before inactivating the enzymes by microwaving at 640 W for one minute three times. Afterward, the leaves were dried in a vacuum drying oven at 60℃ for 2 h, crushed, vacuum-packed, and stored in an ultra-low temperature refrigerator until analysis. 20 g of *B. fargesii* bamboo leaf powder was mixed with 500 mL ethyl acetate, extracted in Soxhlet at 90℃ until colorless, and evaporated to dryness under reduced pressure. We measured moisture content of each sample with an infrared moisture analyzer (OHAUS, Pine, NJ, USA) before extraction, and calculated results based on dry materials.

Determination of macronutrients and organic elements

We selected crude protein (CP), ether extract (EE) and crude fiber (CF) as indicators of bamboo leaf nutrients (Pond et al. 2004). We also determined gross energy (GE), Carbon (C), Nitrogen (N) and Hydrogen (H) as other factors that could influence giant pandas foraging behavior (Nie et al. 2015b). CP was determined by the Kjeldahl method (Conklin-Brittain et al. 1999) using an auto Kjeldahl system (HANON, China), EE was determined with a full automatic soxhlet extractor (HANON, China), and CF analysis was determined by acid and alkali elimination (Jung 1995) through an auto fiber analyser (ANKOM, USA). We comminuted and filtrated (20–40 mesh) bamboo leaves and extracted 10 g of powder for 1 h by 100 mL of 30% (v/v) ethanol aqueous solution using the hot reflux method, the filtrate was then isolated by membrane filtration to remove macro- and micro-molecular components such as polysaccharides and minerals and bamboo leaves. A concentrated solution was obtained after vacuum concentration and submitted for analysis (Zhang et al. 2008).

Determination of PSCs in BLE

We used $NaNO_2$- $Al(NO_3)_3$- NaOH colorimetric method (China Pharmacopoeia Commission, 2010) to analyze the content of total flavonoids (TF) (Wang et al. 2020), Reinecke's salt colorimetric method to measure the content of total alkaloids (TA) (Ren et al. 2012), and high-performance liquid chromatography (Agilent, USA) using tannin acid standard (RONGHE, China) as a control to determine the content of total tannin (TAN)(Popov et al. 2003). GE was measured according to the operating manual using an oxygen bomb calorimeter (PARR 6400, Parr Company). We used an Elemental Analyzer (Euro EA 3000, Leeman Labs, Inc.) to determine the total content of C, N and H.

Estimation of biological activity of BLE

Antioxidant activities

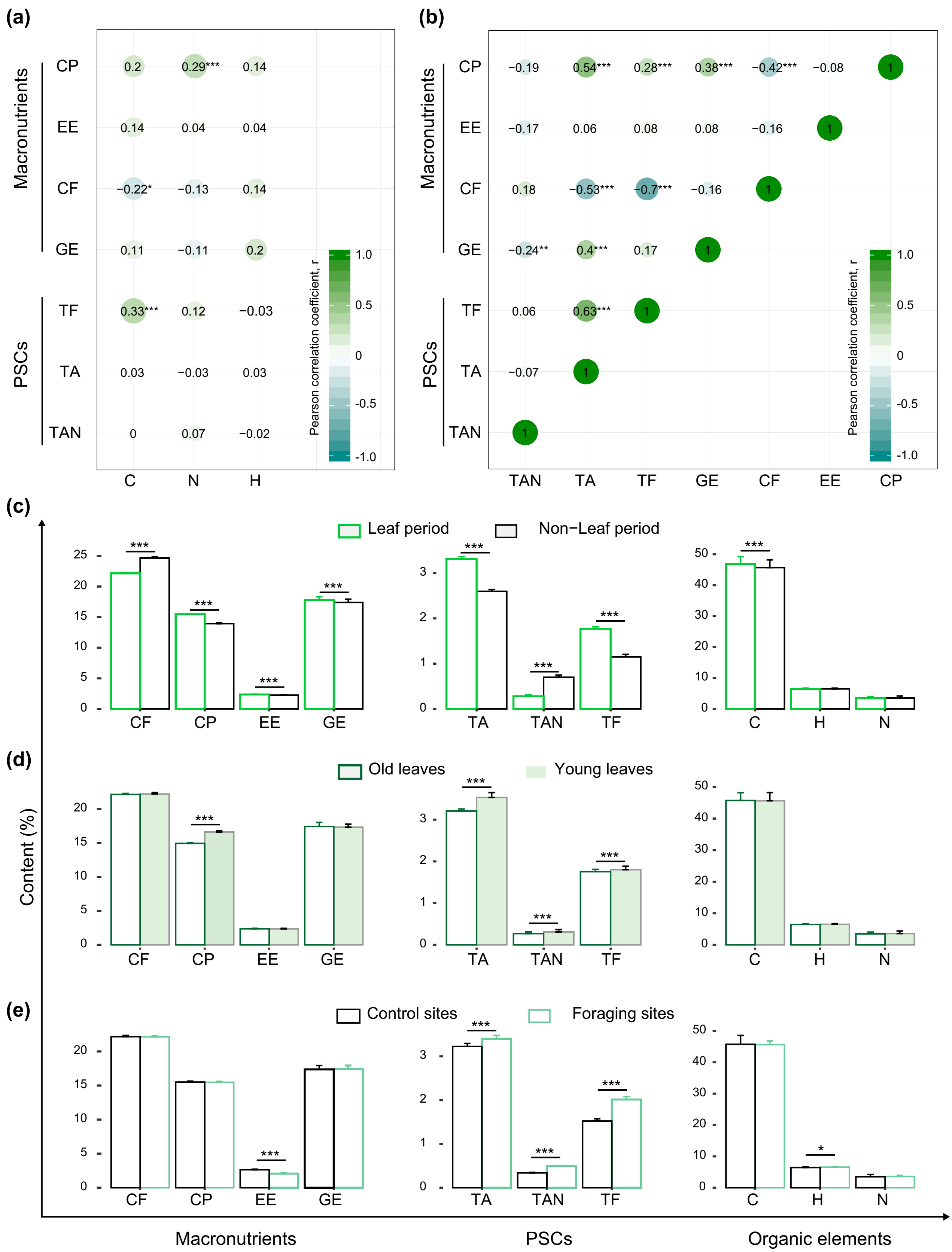
Determining the scavenging effect of the sample on DPPH free radicals is a widely used method to measure antioxidant effects (Brandwilliams et al. 1995). However, because the rate of reaction varies widely among substrates, we set a time gradient for optimizing the method (Molyneux 2004). The absorbance at 518 nm was measured after mixing 200 μL of bamboo leaf extracts (at a different concentration) with 800 μL DPPH solution (6.5×10^{-4} mol L^{-1}) and the system is stabilized, using a microplate reader (Beijing Purkinje General Instrument Co, Ltd, Beijing, China), and 95% ethanol as a blank. Triplicate tubes were prepared for each extract. The DPPH radical scavenging rate (%) was calculated as follows: % radical scavenging rate $= (A_{blank} - A_{sample})/A_{blank} \times 100$. Where A_{blank} is the absorbance of the blank reaction, and A_{sample} is the absorbance of the test compounds. The results were expressed as IC_{50} of DPPH radical scavenging, where IC_{50} means the amount of antioxidant necessary to decrease the initial concentration of DPPH radical ($6.\ 5 \times 10^{-4}$ mol L^{-}) by 50%. The smaller the value of IC_{50} is, the stronger the antioxidant activity of the sample.

Antibacterial activities

Bacterial gastrointestinal disease in giant pandas is a known health concern and cause of death in giant pandas (Qiu and Mainka 1993). We used six standard strains from National Center for Medical Culture Collections (CMCC) and American Type Culture Collection (ATCC) to detect the antibacterial activities of BLE: *Klebsiella pneumoniae* (CMCC (B) 46117), *Salmonella enterica arizonae* (CMCC (B) 47001), *Escherichia coli* (ATCC 25922), *Salmonella enteritidis* (CMCC (B) 50335), *Pseudomonas aeruginosa* (ATCC 27853) and *Yersinia enterocolitica* (CMCC (B) 52204). 6 μL of a 95% ethanol dilution of 125 mg/mL BLE was deposited on a 6-mm diameter filter paper and dried under sterile conditions at 40 °C. The antibacterial activity of BLE was determined by the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI 2019).

Statistical analysis

We calculated the elevational distribution of giant panda occurrence in 100 m increments from 1200 to 1900 m in the *B. fargesii* range binned into the four seasons of spring, summer, autumn and winter seasons (Wei et al. 2015b), and used ANOVA to analyze the proportion of panda occurrences during the Leaf period


(a)
(b)
Macronutrients
PSCs
CP
EE
CF
GE
TF
TA
TAN
C
N
H
Pearson correlation coefficient, r
1.0
0.5
0
-0.5
-1.0
CP
EE
CF
GE
TAN
TA
TF
(c)
Leaf period
Non−Leaf period
(d)
Old leaves
Young leaves
(e)
Control sites
Foraging sites
Content (%)
Macronutrients
PSCs
Organic elements

◂**Fig. 2** Variation and correlation of macronutrients and PSCs in giant panda food resources. **a** The correlation between seven compounds and three organic elements. **b** The correlation between seven compounds. **c** Differences in nutrient and PSCs in *B. fargesii* leaves between the leaf period and non-leaf period; **d** variation in nutritional composition in *B. fargesii* leaves during the Leaf period in young (≤ 1 year) and old (≥ 3 year) bamboo; **b** Variation in nutritional composition in *B. fargesii* leaves in areas where pandas have been known to forage (foraging sites) and areas without signs of panda foraging (control sites). CP, crude protein; EE, ether extract; CF, crude fiber; GE, gross energy; TF, total flavonoids; TA, total alkaloids; TAN, total tannins; C, carbon; N, nitrogen; H, hydrogen. *, ** and *** indicate significant differences between treatment means at $p<0.05$, $p<0.01$ and $p<0.001$, respectively

(September-April) for each elevation gradient. We used independent samples *t* tests to detect the differences in BLE ingredients and biological activity between Leaf vs. Non-Leaf period, young leaves vs. old leaves during the Leaf period, and foraging sites vs. control sites. Levene's Test was used to test for equality of variances, and where the null hypothesis of equal variances was rejected, we applied a Wilcoxon rank sum test that does not assume equal variances. In addition, the Pearson correlation coefficient was used to assess the correlation between the PSCs and nutritional components, as well as among these ingredients which were corrected for multiple testing using the R package psych.

To determine how bamboo PSCs influenced giant panda foraging decisions, we use two statistical analysis methods: multiple stepwise regression analysis and logistic regression. The use preference of altitudinal utilization was defined as the responding variable, and the contents of both macronutrients and PSCs were used as explanatory variables. To further delve into the biological activities and their association with the ingredients, we employed multiple linear analysis. This allowed us to differentiate and understand the contribution of each ingredient in influencing the biological activities observed in bamboo leaves. By utilizing regression equations, we were able to identify the significant factors that influenced both the biological activity of bamboo leaves and the foraging site preferences of giant pandas. This analytical method provides a logical framework to discern the intricate relationships between various factors in the decision-making processes of giant pandas during foraging. All the above analyses and data visualization were processed in R (v. 3.5.1, R Core Team 2018) in which all the significance levels were set at 0.05.

Results

Seasonal variation of elevational preference

GPS data confirmed that pandas occurred primarily in areas below 2000 m during spring and winter, with increasing use of elevations above 2000 m in summer and fall (Fig. 1c). During the Leaf period, giant panda locations were concentrated at 1600–1800 m. Pandas were more likely to be located at 1600 m, 1700 m and 1800 m than other elevations ($p<0.05$, Fig. 1d). To the extent that the movements of these four pandas reflect those of other pandas in this population, these results confirm previous observations indicating that pandas are mostly reliant on *B. fargesii* occurring at these elevations. Thus, we conclude that our analyses of bamboo selection and nutrient content below are valid in that pandas are selecting these elevations seasonally based on access to *B. fargesii*.

Variation of macronutrients and PSCs in *B. fargesii* leaves as a function of season, bamboo age, and selection by pandas

Because we were interested in examining variation in ten dietary covariates, including four macronutrients (CF, CP, EE, GE), three PSCs (TAN, TF, TA) and three organic elements (C, H, N), we first sought to determine if they were intercorrelated. Correlation analyses revealed that the content of CP was remarkably correlated with N ($p<0.001$, Fig. 2a). The contents of CF and TF were markedly related to the C ($p<0.001$, Fig. 2a). In addition, many remarkable correlations between macronutrients and PSCs were detected ($p<0.001$, Fig. 2a), suggesting that these compounds may have collaborative influence on the giant panda foraging strategy. For example, the giant panda prefers bamboos with higher protein and lower cellulose. Here the content of CF was negatively related to the TF ($r=-0.7$, $p<0.001$, Fig. 2b) and TA ($r=-0.53$, $p<0.001$, Fig. 2b), showing the beneficial potentials of TF and TA in the giant panda's foraging decisions.

Season influenced the concentration of several nutrients in *B. fargesii* leaves. Leaves during the Leaf period had significantly higher CP, EE, TF, TA, C and GE and significantly lower CF and TAN ($p<0.001$, Fig. 2c). Comparisons of nutritional composition between young (≤ 1 year) and old (≥ 3 year) *B. fargesii* leaves during the Leaf period revealed that CP, TF, TA and TAN concentrations in young *B. fargesii* leaves were significantly higher than those in old leaves ($p<0.001$, Fig. 2d). However, when we restricted our analysis on the effects of bamboo age only to bamboo collected at foraging sites (i.e., the most biologically relevant comparison), we found that young bamboo was higher in TF, TA and TAN ($p < 0.001$, Fig. 2d). During the Leaf period pandas selected areas to forage on *B. fargesii* leaves that were high in TF ($p < 0.001$), TA ($p < 0.001$) and TAN ($p < 0.001$) concentration (Fig. 2e). Furthermore, for young leaves, the analysis indicates that levels of CP ($p < 0.001$), TF ($p < 0.001$), TA ($p < 0.001$), positively influenced selection by pandas. In addition, CP in old leaves was found to have a significant positive effect on foraging preference ($p < 0.001$, Fig. 2d).

Comparison of anti-oxidants and antibacterial effects in *B. fargesii* leaves as a function of season, bamboo age, and selection by pandas

DPPH free radical scavenging effects analyses demonstrated that compared with the old leaves, young leaves of *B. fargesii* showed significantly higher antioxidant activities ($p < 0.001$, Fig. 3a). The antioxidant activities in bamboo leaves from foraging sites were significantly higher than control sites during the Leaf period ($p < 0.001$, Fig. 3b).

The inhibition zone tests found that old leaves and young leaves of *B. fargesii* showed differential antibacterial effects against six bacteria. For the *K. pneumoniae*, *S. enteritidis* and *Y. enterocolitica*, old leaves showed stronger inhibitory effects; and for the *S. enterica* and *P. aeruginosa*, younger leaves showed much better bacteriostatic efficacy ($p < 0.001$, Fig. 3c). leaf samples collected at giant panda foraging sites differed significantly from those collected in areas without giant panda sign with regard to antibacterial effects ($p < 0.001$, Fig. 3d). Four bacteria including the *K. pneumoniae*, *S. enteritidis*, *Y. enterocolitica* and *E. coli* were more susceptible to leaves of foraging sites than non-foraging sites, while *S. enterica* and *P. aeruginosa* were very sensitive to leaves from non-foraging sites ($p < 0.001$, Fig. 3d).

Effects of macronutrients and PSCs on foraging preference

Our multivariate analysis indicates that both the macronutrients and PSCs influenced the foraging decisions of giant pandas. The multiple stepwise regression identified three macronutrients (EE, CF and GE) and one PSC (TAN) showing markedly effects on the foraging preference, and the logistic regression discriminated one macronutrient (EE) and one PSC (TAN) with significant effects on the foraging preference (Table 1). We found EE and TAN were detected repeatedly in the two models. Next, the multivariate linear model showed the inhibitory effects on the six selected bacteria were mainly caused by TA and TAN (Table 2). TAN content was the only PSC contributing significantly to the inhibition of *K. pneumoniae* and TA was the only PSC contributing to the inhibition of *S. enterica*, *P. aeruginosa,* and *Y. enterocolitica*. TA content and TAN both contributed significantly to the inhibition of *E. coli* and *S. enteritidis.* The only effect mediated by TF was anti-oxidant property.

Discussion

While there is a growing body of research on the health-promoting role of PSCs in humans, the potential for beneficial effects in animals is little studied (Iason 2005; Raubenheimer et al. 2009). Our findings for the giant panda indicate positive selection for several PSCs and PSC-mediated biological activities in *B. fargesii*, suggesting a potential role for PSCs in giant panda foraging selection.

Our findings for PSCs in the panda's foraging habitat paint a picture suggesting foraging strategies designed to increase the consumption of many PSCs, and with the possible exception of tannins during the Leaf period, provide no indication of PSC avoidance. While we cannot rule out the possibility that inter-correlations with some other unmeasured nutrients have influenced our results, these findings suggest the intriguing possibility that pandas (sometimes) seek out bamboo with high concentrations of some PSCs because they provide some beneficial effects, which potentially include controlling parasites, protecting against cancer, improved cardiovascular health, and disease prevention (Min et al. 2003; Athanasiadou and Kyriazakis 2004; Panche et al. 2016; Huang et al. 2018).

Potential role of PSCs in bamboo selected by pandas

As discussed above, each class of PSCs we examined are known to have some beneficial biological activity for humans or animals. Although we cannot evaluate the role of specific compounds consumed by giant pandas, we can infer possible biological function from studies of the same classes of PSCs we evaluated. Flavonoids were the class of PSCs for which we had the most robust evidence for selection by pandas. Although not as well studied as some other PSCs, especially in animals, research has shown that some flavonoids such as quercetin can act as antioxidants by scavenging free radicals produced as a waste byproduct during the oxidative metabolic process (Boots et al. 2008; Egert et al. 2009; Orrego et al. 2009). The removal of free radicals by antioxidants reduces cell damage caused by oxidative stress and can improve health and prolong life through a variety of pathways. Antioxidants may be particularly important in environments undergoing rapid change due to climatic or other anthropogenic factors, as stressors can increase the production of free radicals. Numerous other health benefits of flavones have also been documented, including

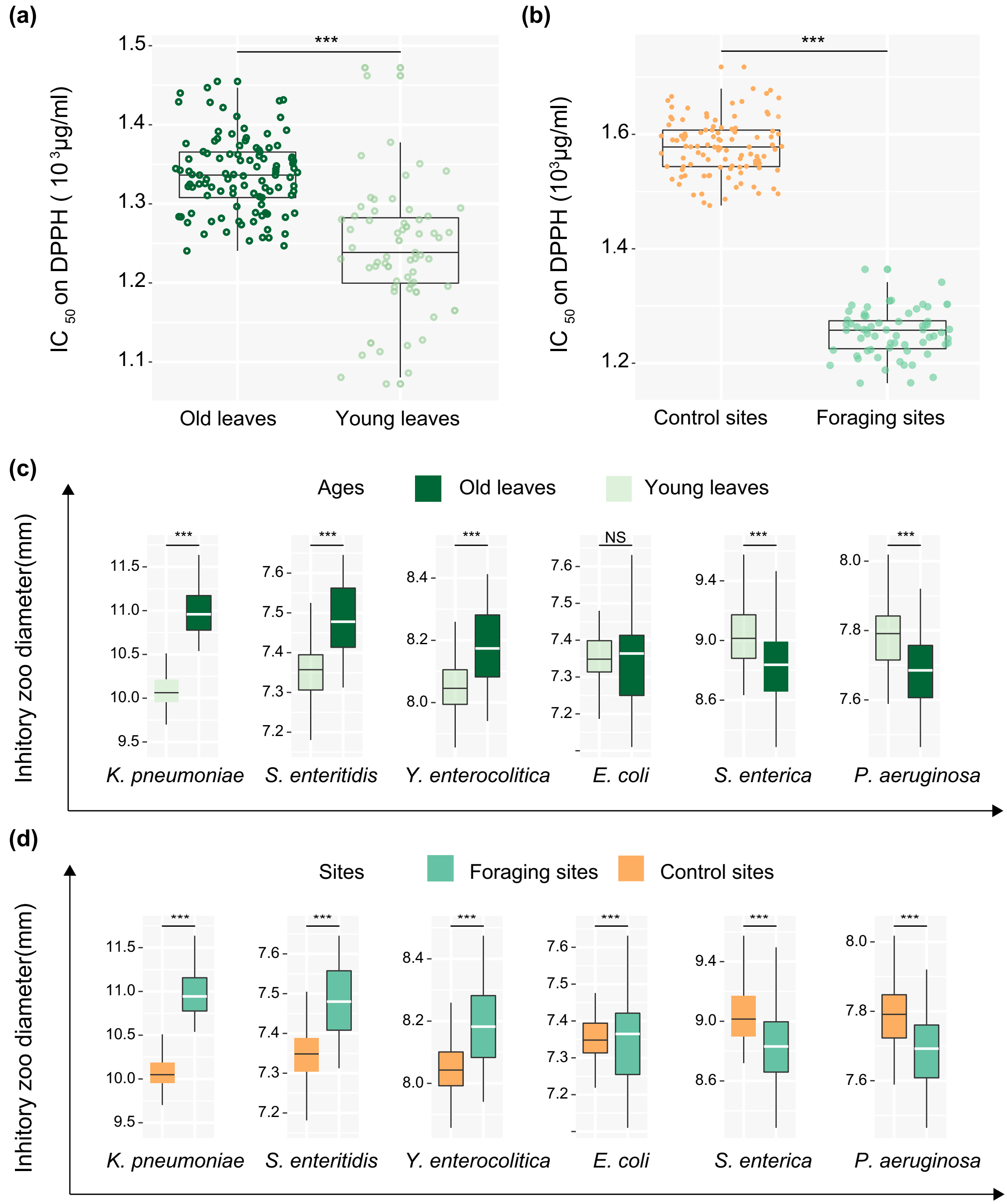


Fig. 3 Variation of biological activities of bamboo leaf extract. **a**, **b** Variation in anti-oxidants in *B. fargesii* leaves in giant panda foraging habitat vs. non-foraging habitat (**a**) and in young vs. old leaves (**b**). IC50 on DPPH: half maximal inhibitory concentration on 2, 2-Diphenyl-1-picrylhydrazyl; the smaller the IC50 value, the better DPPH radical scavenging effect. **c**, **d** Variation in antibacterial effects in *B. fargesii* leaves in young vs. old leaves (**c**) and giant panda foraging sites vs. control sites (**d**). *, ** and *** indicate significant differences between treatment means at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. The larger the inhibitory zone diameter, the better antibacterial effects

anti-microbial (Cottiglia et al. 2001), anti-viral (Li et al. 2002), anti-parasitic (Mitra et al. 2000), and anti-inflammatory (Odontuya et al. 2005). Interestingly, flavonoids produce a bitter taste, and genomic research indicates positive selection for the bitter receptor gene in the Qinling Mountain panda population which we studied here (Roland et al. 2013; Zhao et al. 2012), raising the possibility that taste reception

Table 1 Regression analysis on the foraging selection of giant pandas

Optimal model		Estimate	Std. error	t	p
Multivariate stepwise regression	EE	−0.764	0.179	−4.279	**<0.001**
	CF	0.225	0.088	2.567	**0.011**
	GE	−0.502	0.272	−1.846	0.067
	TAN	9.076	1.064	8.533	**<0.001**
Optimal model		Estimate	Std. error	z	p
Logistic regression	EE	−1.033	0.273	−3.787	**<0.001**
	TAN	9.047	1.545	5.856	**<0.001**

EE ether extract, *CF* crude fiber, *GE* gross energy, *TAN* total tannins. Significant effects are given in bold

Table 2 Effects of three classes of PSCs on biological activity of bamboo leaf extract

Biological activity		Coefficient			Constant	R^2	F	p
		TF	TA	TAN				
Antibacterial property (Inhibitory zone diameter)	*K. pneumoniae*	–	–	18.332	3.424	0.508	21.656	**<0.001**
	E. coli	–	−1.341	6.791	11.143	0.593	10.213	**0.002**
	S. enteritidis	–	−0.908	3.426	9.965	0.392	13.858	**<0.001**
	S. enterica arizonae	–	22.252	–	−64.844	0.441	22.252	**0.019**
	P. aeruginosa	–	0.712	–	5.080	0.495	41.140	**<0.001**
	Y. enterocolitica	–	0.808	–	5.11	0.122	11.080	**0.001**
Anti-oxidant property	IC50 on DPPH	−341.442	−474.75	–	3954.766	0.798	39.601	**<0.001**

TF total flavonoids, *TA* total alkaloids, *TAN* total tannins. "–" indicates this effect was insignificant. Significant effects are given in bold

in pandas has been modified to enhance selection of food resources containing flavonoids.

For tannins, most known biological effects are negative (Iason 2005), yet increasing evidence suggests that low doses of some tannins can be beneficial. Research with herbivorous livestock indicates enhanced anti-parasitic, immune-enhancing, anti-inflammatory, antibacterial, antiviral, and antioxidant functions for tannins (Huang et al. 2018). For tannins, it seems clear that high doses can be toxic, for example interfering with protein digestion, but some evidence indicates that animals benefit from selecting tannins in low doses (Min et al. 2003; Athanasiadou and Kyriazakis 2004; Huang et al. 2018). In a controlled experiment with roe deer (*Capreolus capreolus*), fawns initially preferred tannin-containing foods, but apparently once some internal homeostatic set point was reached, shifted their preference to low doses (Clauss et al. 2003). Many herbivores appear to have evolved physiological mechanisms for countering the toxic effects of tannins, allowing them to consume more of the plant or perhaps facilitating possible beneficial effects of consumed tannins. Mammalian herbivores as diverse as beavers (*Castor canadensis*) and mule deer (*Odocoileus hemionus*) produce salivary proteins that bind to tannins and enhance digestibility (Hagerman and Robbins 1993). At low concentrations, tannins can enhance protein digestion (Barry and Manley 1986; Barry et al. 1986) or protect against intestinal parasites (Min et al. 2003; Marley et al. 2003). In our study, in vitro experimentation indicated that bamboo with high tannins had more antibacterial activity. The mixed biological effects of tannins are reflected in panda foraging behavior: although tannins were found in lower concentrations during the Leaf period when pandas are consuming more leaf, tannins were also higher in younger leaves, which are preferred by pandas. In general, pandas were found foraging in patches higher in tannins than control sites, but when consuming young leaves they selected sites that had lower tannin levels. Because young leaves are higher in tannins, tannin concentrations may exceed levels that are biologically beneficial, which may explain why pandas appeared to avoid tannins when consuming the otherwise more nutritious younger leaves.

The possible beneficial effects of alkaloids are less well studied, but an anti-parasitic function has been documented. Isoquinoline alkaloids are effective against gastrointestinal parasites (Athanasiadou and Kyriazakis 2004). Some infected insect herbivores increase the ingestion of pyrrolizidine alkaloids as an apparent treatment for parasite infection (Villalba and Provenza 2007).

Foraging strategies to balance dynamic and complex nutrient needs

Diet selection is the complex outcome of a foraging animal attempting to meet many competing needs that are constantly changing in response to nutrient availability, the animal's physiological status, whether the animal is within homeostatic boundaries, parasite and disease threats, and other factors (Iason and Villalba 2006; Villalba and Provenza 2007; Raubenheimer et al. 2009). We know that pandas select for a number of important nutrients, including protein, calcium and phosphorus, and they select against cellulose (fiber) (Wei et al. 1999, 2015b; Nie et al. 2015b). Our data reveal that pandas also show signs of positive selection for several PSCs and antibacterial and anti-oxidant activity, as well as more traditional nutrients such as protein, fat and low fiber.

Our study suggests that self-medication may be one of the functions guiding panda foraging strategies. Self-medication has now been documented in a wide variety of vertebrate and invertebrate species (de Roode et al. 2013). Self-medication includes cases where animals seek out plants that are not typically consumed or increased PSC ingestion in plants already in their diet. These behaviors may be observed therapeutically in response to infestation or prophylactically when risk from parasites is high. We cannot know whether giant pandas employ one of these strategies, but their patterns of bamboo selection serving to increase PSC ingestion at low levels indicates that some animals at some times are likely selecting food resources as part of a self-medication strategy, perhaps extending beyond anti-parasitic function to other health benefits.

We suggest that foraging giant pandas select their diet to optimize energy intake, balance nutrient intake for growth, reproduction and maintenance, and at times seek out PSCs as part of a self-medication and health enhancement function. Our findings indicate positive selection for PSCs but effect sizes are relatively small, probably because pandas balance many competing needs when foraging, and increase PSC intake only when challenged with parasites, disease, or other health issues.

Conclusion

As a first attempt to understand the role of plant secondary compounds in giant panda foraging decisions, our results should stimulate additional research to validate and extend our findings. Although more commonly studied with regard to anti-herbivore defenses, PSCs are thought to play a significant role in consumer health. We found that foraging sites not only had higher levels of several PSCs than control sites but also bamboo extract from foraging sites had higher anti-oxidant and antibacterial action. The leaves from young bamboo preferred by pandas also had higher antibacterial action than older bamboo. We suggest that these antibacterial and anti-oxidant effects of bamboo are mediated by PSCs and indeed found that tannins and alkaloids were the main factors predicting antibacterial activity.

As ours was not a controlled study in captivity, we consider this a first and necessary step increasing understanding of the potential role of PSCs in panda nutritional ecology. Because of these constraints working with wild pandas, we are able to document only broad preferences regarding PSC ingestion across time and space and are unable to determine whether individual pandas adjust their consumption of specific PSCs to keep levels in balance (homeostasis) or as self-medication based on disease or parasite status. Because we do not expect pandas to always maximize ingestion of PSCs and instead regulate PSC level based on past consumption or disease challenge, we believe the preferences documented here represent averages including pandas that did not select on the basis of PSC, or selected against them, and pandas that actively selected bamboo high in PSCs to meet some nutritional or biological need.

Although analyses of foraging habitat figure prominently in giant panda management and conservation planning (Wei et al. 2015a; Hong et al. 2016; Hull et al. 2016; Swaisgood et al. 2016; Kong et al. 2017; Xu et al. 2017; Tian et al. 2019), investigations detailing nutrient acquisition strategies are notably lacking and most of those have focused on energy and protein. While these nutrients are clearly important for pandas and govern many aspects of their foraging ecology, more research is needed to address other important nutrients and PSCs in the panda's diet. All bamboo is not equal, and a comprehensive understanding of the panda nutritional strategy will provide better guidelines for managers and decision-makers to preserve and manage habitat to ensure all habitat requirements are met for this conservation icon.

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Author contributions SY, MAO, ZZ and RRS conceived and designed the experiments. TF and YL performed the fieldwork. FY and YD analyzed the data. FY wrote the manuscript and LW revised it. All authors read and approved the final manuscript.

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Availability of data and materials All data produced from this study are provided in this manuscript or will be available from the corresponding author upon reasonable request.

Code availability Not applicable.

Declarations

Competing interests The authors declare no competing financial interests.

Ethics approval Fieldwork was conducted with the permission of the law of the People's Republic of China on the protection of wildlife. The giant pandas involved in this study are approved by the National Forestry and Grassland Bureau.

Consent to participate Not applicable.

Consent for publication Not applicable.

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