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PBDEs (polybrominated diphenyl ethers) pose a risk to captive giant pandas

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Abstract

The Qinling subspecies of giant panda (*Ailuropoda melanoleuca qinlingensis*) is highly endangered; fewer than 350 individuals still inhabit the Qinling Mountains. Previous research revealed that captive pandas were exposed to bromine, so we hypothesized that captive pandas also were exposed to, and affected by, polybrominated diphenyl ethers (PBDEs). To test this hypothesis, we sampled blood and feces of captive and wild pandas, their drinking water, food (bamboo leaves) from the Shaanxi Wild Animal Research Center (SWARC) and the Foping National Nature Reserve (FNNR), and supplemental feedstuff fed to captive pandas at SWARC. We found 13 congeners of PBDEs in fecal samples, of which BDE47, BDE66, BDE71, BDE99, and BDE154 predominated; total PBDE concentration in feces of captive pandas was 255%

higher than in wild pandas. We found nine PBDEs congeners in blood samples: BDE153 and BDE183 predominated, and concentrations of PBDEs in blood from captive pandas also were significantly higher than in wild pandas. The primary source of PBDEs appears to be the bamboo fed to the pandas: total concentration of PBDEs were 5473 and 4835 pg·g^{-1} in the bamboo *Fargesia qinlingensis*; 2192 and 1414 pg·g^{-1} in the bamboo *Bashannia fargesii*; 0.066, 0.038 pg·mL^{-1} in drinking water; and 28.8 pg·g^{-1} in supplemental feedstuff for captive and wild pandas, respectively. BDE99 and BDE47 could threaten the health of captive pandas, whereas other PBDE congeners may pose additional health risks to the captive pandas. In the short term, this risk may be ameliorated by strict control of food quality. In the long term, however, reducing air, water and soil contamination to improve environmental quality will best reduce these risks.

Keywords: PBDEs; Captive Panda; Feces and Blood; Food; Health risk

Capsule: Captive pandas were exposed to higher concentrations of toxic PBDEs than wild pandas. PBDEs are most prevalent in the bamboo fed to the pandas, highlighting the need for quality control on the food supply of captive pandas.

Introduction

The giant panda (*Ailuropoda melanoleuca*) is one of the rarest animals in the world. Approximately 1800 individuals remain in anthropogenically fragmented habitats (SFA, 2015), of which < 350 individuals are of the Qinling subspecies (*A. melanoleuca qinlingensis*) living in the

Qinling Mountains of China (SFA, 2015). In the last several decades, two strategies have been used to protect this species. One strategy is *ex-situ* breeding in, for example, the Beijing Zoo, the Wolong Breeding Center, and the Shaanxi Wild Animal Research Center (SWARC). The other strategy is the establishment of natural conservation zones to preserve panda habitat. In the last several decades, 67 conservation zones, with a total area > 43,600 km^2, have been established (SFA, 2015).

It is generally assumed that captive breeding centers can effectively protect giant pandas from the adverse impacts of human activities. However, canine distemper virus has killed at least four pandas at SWARC (Mara, 2015) suggesting that new measures are needed to protect captive individuals of this iconic endangered species. Environmental pollution further stresses captive animals. For example, we have shown that captive pandas are exposed to heavy metals including cadmium, zinc, chromium, arsenic and lead (Chen et al., 2016). We also found that chlorine and bromine were 690% and 330% higher in feces of captive pandas than in those of wild pandas (Chen and Ma, 2017), and we therefore hypothesized that captive pandas may be exposed to and affected by polybrominated diphenyl ethers (PBDEs).

PBDEs are brominated flame retardants that are used in electronic equipment, textiles, cabinets for television and computers, and in many plastic products (WHO, 1994; Darnerud et al., 2001; Kim et al., 2012). PBDEs are lipophilic, are released slowly into the environment, can bioaccumulate in tissues of humans and other mammals, and are toxic to them (Hooper and McDonald, 2000; De Wit, 2002; Hu et al., 2008). Exposure of laboratory animals to high concentrations of PBDEs can suppress production of antibodies and proliferation of lymphocytes

(Darnerud and Thuvander, 1998), decrease thymic weights (Fowles et al., 1994), cause immunomodulatory turbulence, and lead to hormonal deficits (Eriksson et al., 2001; Branchi et al., 2003). Modulating effects of PBDE exposure on endocrine systems of wild animal also have been documented (Legler and Brouwer, 2003; Darnerud, 2003). Recent research showed that giant pandas were exposed to PCDDs, PCDFs, PCBs, and heavy metals from the bamboo they eat (*Fargesia qinlingensis* and *Bashania fargesii*) in both captive breeding centers and *in situ* in conservation areas (Chen et al., 2016). However, there has been no research on exposure of captive or wild pandas to PBDEs, or their concentrations in panda feces and blood.

The objective of this study was to (1) test whether captive or wild pandas are exposed to PBDEs. (2) document and compare the concentrations of PBDEs in wild and captive pandas; and (3) identify possible sources of PBDEs contamination. Feces, drinking water, and food (bamboo) were collected from SWARC and the Foping National Nature Reserve (FNNR), and blood samples and supplemental feedstuff were sampled at SWARC (Fig.1).

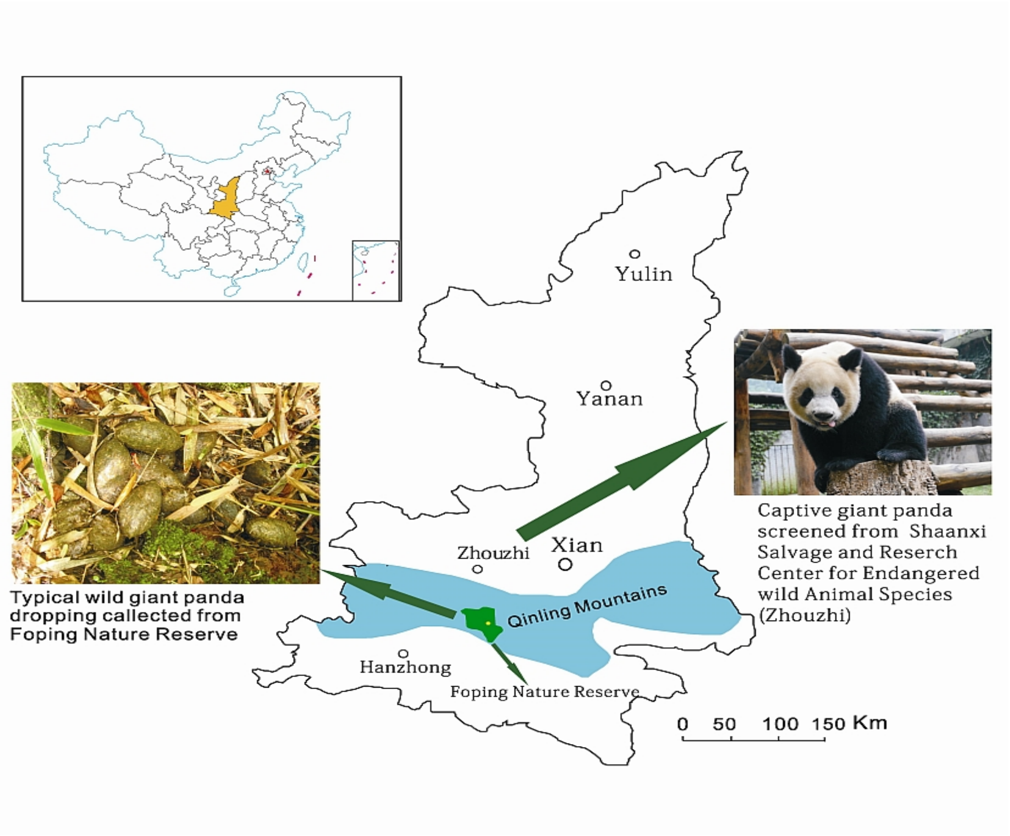

Figure 1. Sample Collection Sites. The Shannxi Wild Animal Research Center (SWARC) is located at 34° 04´ N, 108°19´ E in Zhouzhi County, Shaanxi province. The Foping National Nature Reserve (green shaded area) is located in the area bounded by 33° 33´ – 33 44´ N, 107° 40´ – 107° 55´ E within the Qinling Mountains (blue shaded area).

Materials and methods

Sample collection

Giant pandas are protected in China, capturing them is illegal, and so samples from wild animals must be collected non-invasively. Fecal samples (droppings: Fig. 1) were collected from 16 different locations within FNNR. Sampling locations were spaced 10-km apart and every four independent samples were pooled into a mixed sample. Droppings of 16 captive pandas were collected from SWARC, which was established in 1987 to conserve the Qinling panda. Each set of 16 fecal samples were pooled for analysis into four samples each consisting of four independent samples.

Fresh leaves of living plants (500 g) of the two bamboo species (*Fargesia qinlingensis, Bashania fargesii*) that are the primary food of the panda were collected in proximity to where the droppings were collected at FNNR and around SWARC. Water samples (500 mL) were collected into Pyrex borosilicate amber glass bottles from streams at FNNR near where we collected the droppings, and from the SWARC water supply. At both FNNR and SWARC, 12 samples of each bamboo species and of freshwater were collected. They subsequently were pooled to produce four mixed samples each consisting of three independent samples from each site. In addition, four samples of mixed feedstuff, provided as a nutrient supplement for captive pandas, also were collected from SWARC.

Finally, blood samples were obtained from three similarly-aged pandas rescued from the Qinling Mountains and three captive pandas bred at SWARC. These blood samples were residuals from regular, routine physical examinations of the individual pandas. Prior to examination, the pandas were anesthetized with 25% ketamine (dosage: 8 $mg{\cdot}kg^{-1}$). After collection, the blood was placed in EDTA tubes and frozen at –80 °C for analysis of PBDEs.

Sample preparation and extraction

PBDE congeners were analyzed using US Environmental Protection Agency (EPA) method 1614 with minor modifications (Li et al., 2008). Bamboo, feces, and feedstuff samples were freeze dried and then homogenized by passing them through a stainless steel sieve (0.5-mm mesh). Each 3-g homogenized sample was spiked with a ^{13}C-labeled surrogate standard (EPA methods 1613B and 1668A) and extracted using accelerated solvent extraction (ASE) for 24 h with dichloromethane (150 mL) and hexane (150 mL) at 55 °C. After ASE, acidic silica (15 g, 30% w/w) was added to the sample to remove lipids. Then, 5 g of anhydrous sodium sulfate was added to the extract. The extract sample was rotary evaporated to 2 ml and then passed through a multi-layered silica-gel column that had been pre-cleaned by hexane (100 mL). After the sample was loaded, the PBDE congeners were eluted with 70 ml hexane followed by 70 mL dichloromethane. The eluant was then concentrated to 2 ml on the rotary evaporator. Its volume was further reduced with a gentle nitrogen flow and the solvent was changed to 20 µL nonane in a minivial.

PBDEs in water samples were extracted using US EPA method 1614. Prior to extraction, 1 L of the liquid samples were filtered using 0.45-µm microporous membranes to remove the particle phase and then spiked with a ^{13}C-labeled BDE-LCS standard. Organic halogen pollutants were adsorbed by siphon, 50 mL of acetone was used to flush the pillars and a 100-mL mixture of acetone and water was collected. Resins were extracted using ASE for 24 h with dichloromethane (300 mL) and hexane (300 mL) at 55 °C. After ASE, a 15-mL acetone-cleaned soxhlet extractor and zeolite was used to obtain a 430-mL solution, which was transferred to a 1000-mL separatory funnel, washed with 100 mL of ultrapure water for 3 times, and extracted with 30-mL n-hexane for 3 times to obtain a clear and transparent organic phase solution. This organic phase solution was evaporated to 2–3 mL and purified on the silica gel column. After adding 50 ml n-hexane and evaporateding to 2–3 mL, we added 15 mL isooctane, evaporated to 2–3 mL and used 6-mL isooctane to clean. The solution was purged by nitrogen stream and diluted to 1 mL in a brown bottle at –20 °C for further analysis.

Instrumental analysis

BDEs 17, 28, 47, 66, 71, 85, 99, 100, 138, 153, 154, 183, and 190 were analyzed using gas chromatography (Agilent 6890, USA) coupled with a high-resolution mass spectrometer (HRMS). The HRMS (Waters Micromass, Manchester, UK) operated in selected ion monitoring (SIM) mode with resolution >10,000. The m/z ratio of all the PBDE congeners was 79 and 81 except for BDE47 (m/z 325 and 327), BDE99 (m/z 404 and 406) and BDE183 (m/z 562 and 564). Exactly 1 µL of the sample was injected with a CTC PAL autosampler in splitless mode into an HB-5 (30 × 250-µm i.d. × 0.1-µm film thickness) capillary column for separation. The flow rate of the carrier gas was 1.2 mL/min and the carrier gas was Helium. The program was as follows: the injector was temperature programmed to ramp from 60 °C to 320 °C at 150 °C/min. The oven started at 80 °C held for 1 min, increased to 200 °C at 10 /min, held at 200 °C for 1 min, increased to 300 °C at 20 °C min, and then held at 300 °C for 5 min. The temperature of the ion source was 150 °C.

Quality assurance and quality control

All solvents were pesticide-residue grade and were purchased from Fisher (Hampton, NH, USA). Silica gel was obtained from Merck (silica gel 60, Darmstadt, Germany). ^{13}C labeled surrogate and labeled injection standards were purchased from Wellington Laboratories (Guelph, Canada).

All analytical procedures were checked by the strict quality assurance and control measures to avoid sample- and cross-contamination. Reference material and 3 blank control samples (ultrapure water) were analyzed using the same methods as described above. Triplicate samples were analyzed to determine repeatability and reproducibility. To monitor analytic losses, all samples were spiked with internal standards of ^{13}C-labeled BDE47, 99, and 153. The mean recoveries of ^{13}C-labeled surrogate PBDE congeners 47, 99 and 153 were in the range of 54.2 ± 12.1%, 66.0 ± 10.1%, 102.2 ± 20.1%, respectively, which were well in the limits according to US EPA Method 1614; all the content of PBDEs in the control (blank) samples were below the limit of detection (LOD), which for BDEs 17, 28, 47, 66, 71, 85, 99, 100, 138, 153, 154, 183, and 190 are 5, 4, 3, 15, 6, 7, 7, 7, 4, 1, 12, and 4 $pg \cdot g^{-1}$, respectively). If concentrations of actual samples fell below LOD, 1/2 of the LOD values were used in subsequent statistical analysis.

Data analysis

Correlation analysis (CA) and principal components analysis (PCA) were used to analyze the association between 13 PBDE congers in different samples. Paired samples were analyzed using *t*-tests. All statistical analyses were done using the IBM statistical package SPSS 20.0 (IBM Corp., USA).

Evaluation methods

The giant panda’s health risk evaluation was calculated using following equations detailed in the Exposure Factors Handbook (US EPA 1997). Assuming that giant pandas only feed on bamboo leaves, average daily dose (ADD) was calculated as:

$$ADD = \frac{C \times IR_S \times EF \times ED}{BW \times AT}$$

where C is concentration of PBDEs (mg/kg), IR_S is the ingestion rate of bamboo, EF is the exposure frequency (350 day/year), ED is the exposure duration (10.36 years), BW is the average body weight (105 kg, from mid-range of 80-130 kg [Zhang and Wei 2006]), and AT is the averaging time (3781.4 days).

Noncancer toxic risk was determined by the model hypothesis of HQ (Hazard Quotient):

$$HQ = \frac{ADD}{RfD_o}$$

where RfD_o is the reference dose of PBDEs (US EPA, 1997). Risk increases with HQ (Hang et al. 2009). If $HQ \leq 1$, risk exposure is relatively safe. If $1 < HQ \leq 10$, considerable threat is suggested, Finally, if $HQ \geq 10$, high chronic risk is suggested.

Results

Concentrations of PBDEs

Total PBDEs concentrations were consistently and significantly greater in captive pandas and their food supply than in wild pandas and their food and water supply (Fig. 2). In the fecal samples, Σ13PBDE of captive pandas was 2.55 times greater than in wild pandas (Fig. 2A). Σ13PBDE of *Fargesia qinlingensis* was 1.13 times higher, and of *Bashania fargesii* 1.55 times higher, in leaves eaten by captive pandas (Figs. 2B, 2C). Water samples had low concentrations of PBDEs (Fig. 2D).

Thirteen congeners of PBDEs were found in fecal samples; BDE47, BDE66, BDE71, BDE99 and BDE154 predominated in captive pandas (Fig. 3A). Of the dozen congeners found in the two bamboo species eaten by captive pandas, BDE47 and BDE99 predominated in *Fargesia qinlingensis* and *Bashania fargesii*, respectively (Figs. 3B, 3C). Although captive pandas were exposed to somewhat higher concentrations of PBDEs in their water supply (Fig. 2D), the concentrations of each congener were quite low and none predominated (Fig. 3D). Ten PBDE congeners were found in the supplemental feedstuff provided for captive pandas, with BDE28 and BDE183 predominating (Fig. 3E). Finally, nine PBDE congeners were found in the blood samples collected from SWARC. BDE153 and BDE183 were the predominant congeners in captive panda blood samples, and occurred in significantly higher concentrations than in blood sampled from wild pandas (Fig. 3F).

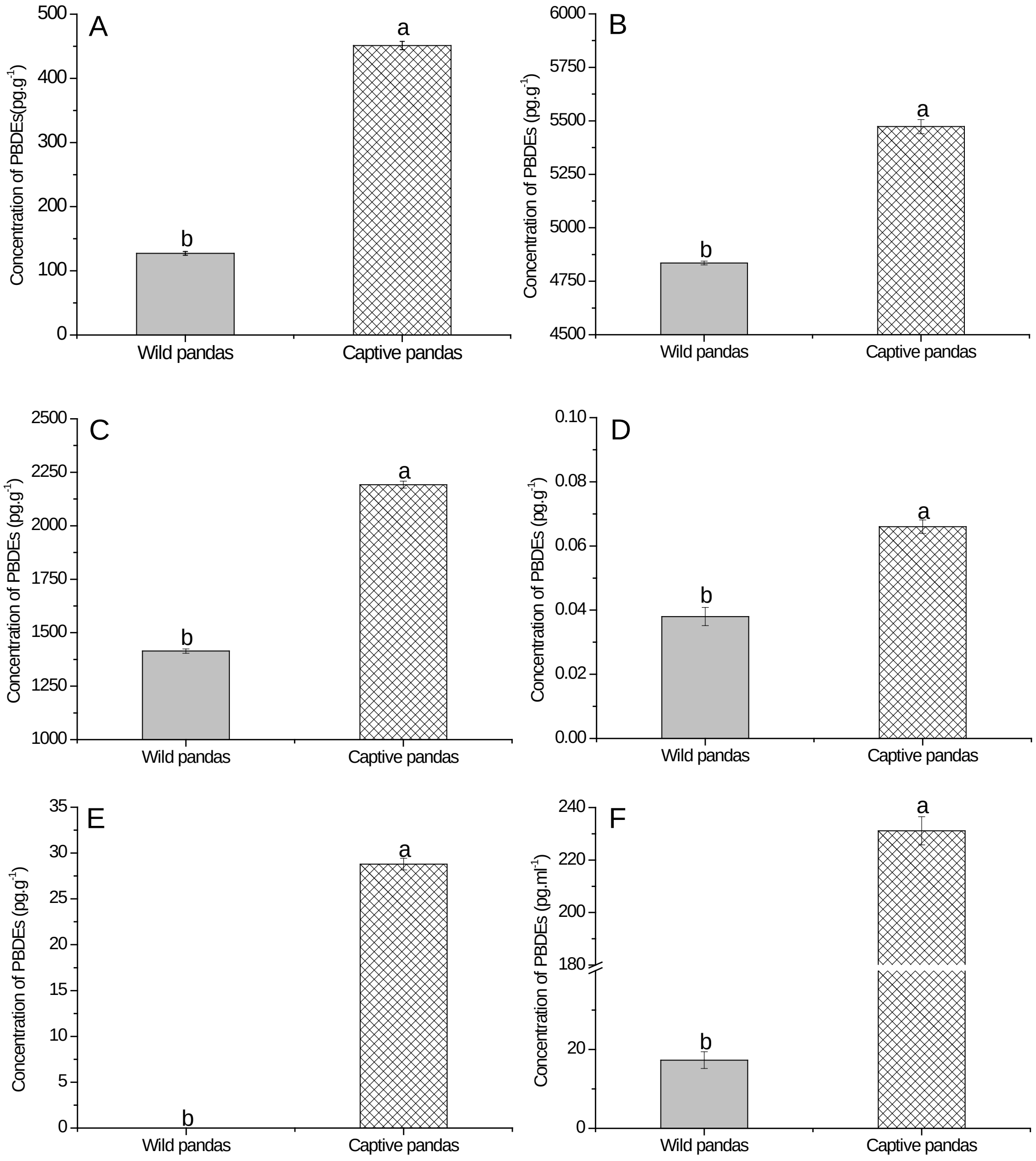

Fig. 2. Total concentrations of PBDEs (Σ13PBDEs) in (A) fecal samples; (B) leaves of *Fargesia qinlingensis*; (C) leaves of *Bashania fargesii;* (D) drinking water; (E) supplemental feedstuff; and (F) blood sample of wild (gray bars) and captive (cross-hatched bars) giant pandas. The concentrations of PBDEs in A, B, C and E are based on dry weight. In (F), the wild pandas were three 17-year old individuals rescued from Qingling and the captive pandas were 8–9-years old. Bars (means ± 1 SE of the mean from n = 4 independent replicates comprising three or four pooled samples). Different letters indicate significant differences between the wild and captive pandas identified using Tukey HSD test (all $P < 0.01$). pg · g^{-1} lw^{-1} = nanograms per gram lipid weights.

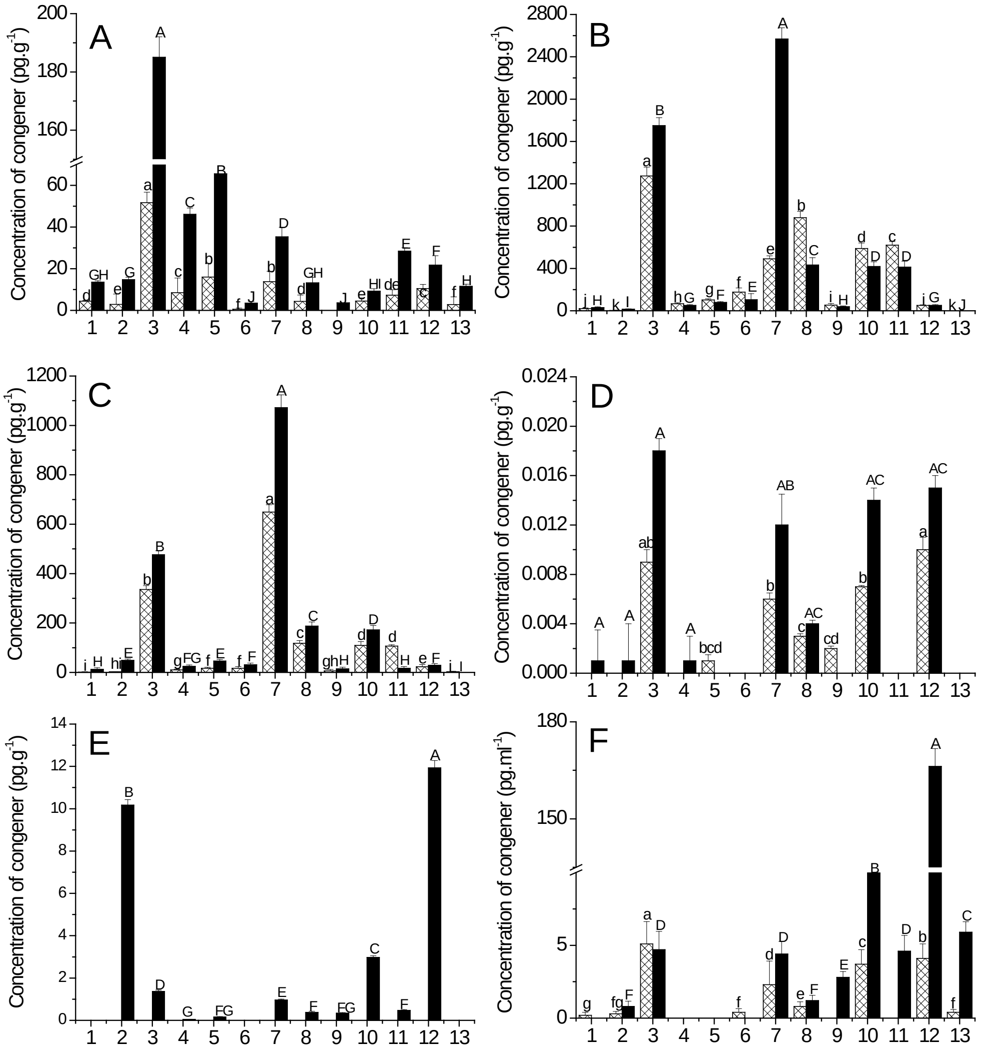

Fig. 3. Concentrations of individual PBDE congeners in (A) feces; (B) leaves of *Fargesia qinlingensis*; leaves of *Bashania fargesii*; (D) drinking water; (E) supplemental feedstuff; and (F) blood from wild (cross-hatched bars) and captive (black bars) giant pandas. Numbers 1 – 13 on the *x*-axis denote different congeners, respectively: BDE17; BDE28; BDE47; BDE66; BDE71; BDE85; BDE99; BDE100; BDE138; BDE153; BDE154; BDE183; BDE190. Different letters indicate significant differences between captive and wild pandas ($P < 0.05$).

Statistical results

The highest positive correlations in Σ13PBDEs were detected for feces and blood *vs.* bamboos ($r > 0.88$), and blood *vs.* feedstuff ($r = 0.88$). Concentration of PBDEs in water samples were not significantly correlated with any of the other samples (Table 1).

Three principal axes accounted for most of the variance in the groupings of PBDE congeners (Fig. 4). In leaves of *Fargesia qinlingensis*, the first three principal axes accounted for 87.8% of the variance (Fig. 4A), whereas they accounted for 62.5% for *Bashania fargesii* (Fig. 4B), and 77.4% in feedstuff (Fig. 4C). Only one grouping was found for PBDEs in drinking water (19.8% of the variance; Fig. 4D). The PCA identified clusters of the congeners BDE47, BDE66, BDE71, BDE99, and BDE154 in leaves of *Bashania fargesii* that matched the predominant congeners found in fecal samples (compare Fig. 4B with Fig. 3A).

Table 1 Spearman correlation matrix for PBDEs measured in captive samples

	Fargesia qinglingensis	*Bashania fargesii*	Blood	Water	Feces
Bashania fargesii	0.89**				
Blood	0.92**	0.92**			
Water	-0.11	-0.10	0.15		
Feces	0.90**	0.88**	0.93**	-0.09	
Feedstuff	0.73**	0.75**	0.88**	0.309	0.79**

**. $P < 0.01$ level (2-tailed test).

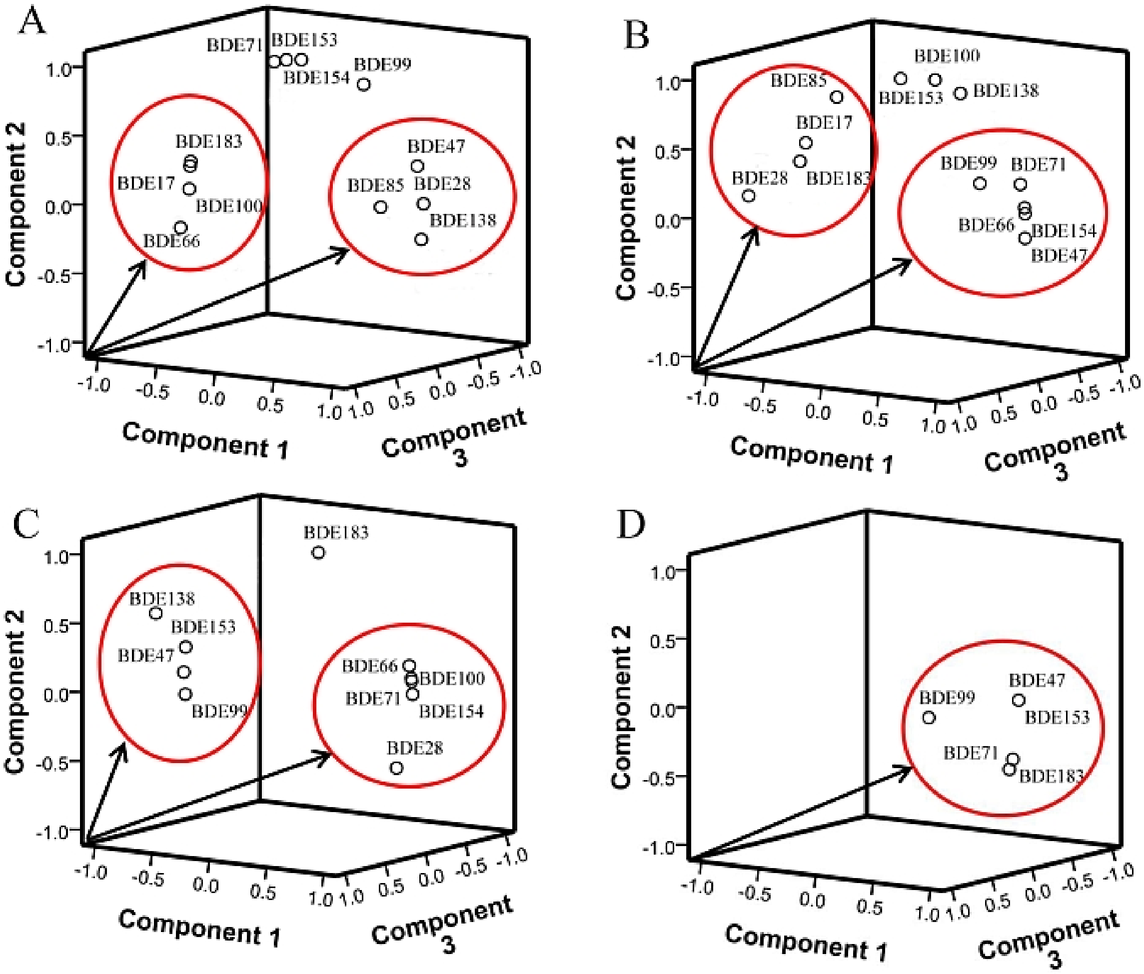

Fig. 4. Principal component triplot (principal axes 1–3) for 13 PBDE congeners in (A) leaves of *Fargesia qinglingensis*; (B) leaves of *Bashania fargesii*; (C) supplemental feedstuff; and (D) drinking water of samples taken at SWARC.

Health risk assessment

The ordering of hazard quotients (*HQ*) of the top five PBDE congeners in captive pandas was BDE99 (1.60) > BDE47 (1.10) > BDE100 (1.07) > BDE153 (0.97) > BDE154 (0.55), and was BDE47 (1.20) > BDE100 (0.99) > BDE154 (0.75) > BDE153 (0.57) > BDE99 (0.55) in wild pandas. with *HQ* values >1, BDE99 and BDE47 appear to pose a significant health risk to captive giant pandas, whereas BDE47 is congener putting wild pandas at risk.

Discussion

The first objective of this study was to test this hypothesis that giant pandas were exposed to PBDEs. Our previous research had found that Br in fecal samples of captive pandas were 3.3 times higher than in wild pandas (Chen et al., 2017), and data reported here supported the resulting hypothesis that captive pandas were exposed to PBDEs (Figs. 2A, 3A). In captive pandas, BDE47, BDE66, BDE71, BDE99, BDE154 were the major PBDE congeners in fecal samples, which matched the profile of PBDEs in leaves of the bamboo *Bashania fargesii* (Fig. 4B). This result suggests that the bamboo that is the primary food supply for pandas is the main source of PBDE exposure. Plants can accumulate high concentrations of toxicants in tissues that subsequently can accumulate and in the animals (Burreau et al. 2006; Voorspoels et al. 2007; McKinney et al. 2011; Krieger et al. 2016). Once in their body, PBDEs can lead to deficiencies in neural responses, thyroid hormone disorders, and carcinogenicity (McDonald 2002; Staskal et al. 2005; Lee et al. 2014).

Many studies have shown that PBDEs bioaccumulate in aquatic (Law et al., 2003) and terrestrial (Huwe et al., 2002; Jaspers et al., 2005; Pirard et al., 2007; Voorspels et al., 2006, 2007; Da Chen et al., 2012, 2013; Crosse et al., 2012; Andersen et al., 2015) species. However, we are aware of fes studies that sampled PBDEs in fecal samples (Zheng et al., 2015), which are an appropriately non-invasive method to sample PBDEs in a rare species like the giant panda.

The second objective of our research was to compare exposure of wild and captive pandas to PBDEs. Not surprisingly, given the anthropogenic origin of PBDEs, the Σ13PBDE was significantly higher in samples taken from captive pandas. Plants often reflect the content of organic pollutants in the environment (Collins and Finnegan, 2010) because the soil-air-plant pathway (pollutants volatilized from soils into the atmosphere are deposited onto plants) describes uptake of organic pollutants from contaminated soils (e.g., Paterson et al., 1991; Trapp and Matthies., 1997; Harrad et al., 2006; Collins and Finnegan., 2010; Ding et al., 2014). We found that the Σ13PBDE of in *Fargesia qinlingensis* and *Bashania fargesii* growing around the captive breeding center was significantly higher than that growing in the nature reserve (Fig. 2B and 2C). The panda captive breeding center at SWARC is located close to the large city of Xi'an and also to several potential sources of PBDE contamination: waste incinerators, electronic-waste processing facilities, and industrial discharges (He et al., 2014; Kosior et al., 2015; Wang et al., 2015).

The final objective of our research was to identify possible sources of exposure for PBDE pollution by pandas. This study is the first to measure PBDE concentrations in bamboo, so we can compare our results only with those from unrelated plant species. The Σ13PBDEs of *Fargesia qinlingensis* and *Bashania fargesii* growing at FNNR and SWARC in our study were higher than that of the moss *Pleurozium schreberi* in uncontaminated (755.6 pg/g dry mass) or urban (3062.9 pg/g dry mass) areas. Pandas eat little besides bamboo. They can consume on average 30 kg/day (Tuanmu et al., 2013) and bamboo accounts for > 99% of their diet (Hu, 1991, 2000), yet only 25% of the nutrients in bamboo can be assimilated (Zhou et al., 2008). This diet, together with the similarities in congener profiles of panda fecal samples (Fig. 3A) and the clustering in the PCA, (Fig. 4B) suggest that bamboo is the primary source of PDBE exposure for pandas.

The very low concentrations of PBDEs in water (Figs. 2D, 3D, 4D) suggest that it is an unlikely source of PBDEs for pandas. Likewise, the feedstuff appears an unlikely route of exposure. In captivity, pandas are fed a steamed bread supplement (“feedstuff”) that includes additional ingredients, including milk powder, apple, carrot, steamed bran, rice flour, maize flour, bean flour, fishmeal, bone meal, and mineral additives that provide supplemental nutrients essential for successful breeding programs (Chen and Ma, 2016). The Σ13PBDE in feedstuff (28.8 pg·g^{-1}; Fig. 2E) exceeded PBDEs concentrations in some farmland grains (13.7 pg·g^{-1}: Luo et al., 2009) but not others (30–440 pg·g^{-1}: Zheng et al., 2015). This might be because the feedstuff used in our research was purchased from local markets instead of having been made on site from locally-grown ingredients.

To our knowledge, this is the first investigation of exposure of pandas to PBDEs, and one of only a very few studies of PBDE exposure and bioaccumulation in a terrestrial species (Hoshi et al., 1998; Christensen et al., 2005). There are few comparables, but the *HQ* model for exposure risk suggests that BDE99, BDE47, and BDE153, each of which has an *HQ* >1, could threaten the health of captive giant pandas.

Pandas in captive breeding centers generally are thought to be better protected from human activities than are wild pandas in nature conservation zones. However, our previous research has shown that captive pandas are exposed to a variety of environmental pollutants (Chen et al. 2016). The data presented here provide further evidence that the habitat and captive breeding centers of giant pandas are polluted by PBDEs, and that exposure to PBDEs is significantly greater in captive conditions.

PBDEs in pandas most likely come from the bamboo they eat. Every giant panda consumes 30 kg of shoots and leaves of bamboo, on average, every day (Tuanmu et al. 2013). Therefore, even relatively low concentration of PBDEs in bamboo can still lead to a high dietary exposure that threatens the health of the giant pandas. For mammals, PBDEs can be transferred to nursing offspring via mother's milk (Travis and Hattermer-Frey, 1991; Darnerud, 2003; Beineke et al., 2005; Beineke et al., 2007). PBDEs also have immunotoxicity and can be immunosuppressants (Arkoosh et al., 2010; Frouin et al., 2010; Lv et al., 2015) that make pandas more vulnerable to bacterial and viral infections.

Conclusions and Recommendations

Our data suggest that pandas are exposed to high levels of PDBEs in captive breeding centers, and may represent a significant health risk for pandas in captivity. We recommend that managers of these centers and captive breeding programs, including the Chinese State Forestry Administration (SFA), seek strategies to minimize PDBE exposure by pandas lest decades of successful *ex situ* conservation efforts become compromised by the increasing pollution associated with Chinese economic development. A short-term solution to addressing this issue is to reduce the supply of contaminated bamboo and to grow uncontaminated bamboo strictly for captive pandas. In the long term, however, sustaining a successful captive breeding program for pandas will require reduction of air, water, and soil pollution that will lead to improvements in the environmental quality of the giant panda's natural habitat.

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