Environmental toxicants impair liver and kidney function and sperm quality of captive pandas

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ABSTRACT

Captive pandas are exposed to higher concentrations of environmental toxins in their food source and from atmospheric pollution than wild pandas. Moreover, the Qinling panda subspecies had significantly higher concentrations of toxic chemicals in its feces. To determine whether these toxicants also accumulate in panda's blood and impair its health, concentrations of persistent organic pollutants (POPs) and heavy metals were measured in blood samples. Four heavy metals (As, Cd, Cr and Pb), PCDD/Fs and PCBs were detected in blood drawn from captive Qinling pandas. Time spent in captivity was a better predictor of toxicant concentration accumulation than was panda age. More than 50% of the studied pandas were outside the normal levels for 11 health parameters, and five (ALT, LDH, Ca, Cl, TB) of the 11 parameters classified as abnormal were correlated with blood pollutant concentrations. The proportion of live sperm was significantly lower and the aberrance ratio of sperm was significantly greater for captive pandas than for wild ones. A short-term solution to reduce the health impacts of pollution and toxicant exposure of Qinling pandas is to relocate breeding centers to less contaminated areas and to strictly control the quality of their food provided. A longer term solution depends on improving air quality by reducing toxic emissions.

1. Introduction

The giant panda (*Ailuropoda melanoleuca*) is one of the most endangered animals in the world, and it is recognized worldwide as a symbol for conservation. Conservation zones and captive breeding centers have been established to protect giant pandas, but our previous work has shown that conservation efforts are being compromised by increasing levels of pollution associated with China's rapid industrialization and urbanization (Chen et al., 2016, 2017). Both the Sichuan and Qinling subspecies of giant panda are exposed to high concentrations of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs), polychlorinated biphenyls (PCBs), chromium (Cr), cadmium (Cd), arsenic (As), and lead (Pb), and captive pandas are exposed to higher concentrations of pollutants than wild pandas (Chen et al., 2016). Moreover, droppings from the Qinling subspecies contain significantly higher concentrations of As, Cd, and Pb than those from the Sichuan subspecies (Chen et al., 2016).

Recently issued data from State Forestry Administration (SFA) showed that there are only 345 remaining individuals of the Qinling subspecies of the giant panda (SFA, 2015), and it is a higher priority for conservation than the Sichuan subspecies. The Shaanxi Wild Animal Research Center (SWARC) (Louguantai, Zhouzhi County, Xi'an city, 34°04′N, 108°19′E) is the only center in China focused on conservation of the Qinling subspecies. There, environmental pollutants (e.g., PCDD/Fs, PCBs, Cd, Cr, As, and Pb) are present in high concentrations in the food fed to the captive pandas (Chen et al., 2016) and atmospheric deposition was the most likely origin of heavy metals and persistent organic pollutants (POPs) in the diets of captive and wild Qinling pandas (Chen et al., 2016).

Persistent organic pollutants and heavy metals are persistent hazardous toxicants that may be transported over long distances in air and water. Both humans and wild animals are vulnerable to these toxicants. For example, PCDD/Fs are associated with developmental toxicity, immunotoxicity, and reproductive toxicity in humans and other animals (Lohmann et al., 2007; Sfriso et al., 2014; Fernandez-Rodriguez et al., 2015). Similarly, PCBs and their breakdown products are known endocrine disrupters, cause the loss of renal cell viability, and are associated with increased risk of chloracne, goiter, anemia, and cancer (Qiu,

2013; Sfriso et al., 2014; Eqani et al., 2015; Fernandez-Rodriguez et al., 2015; Gustavson et al., 2015). Heavy-metal exposure has been linked with increased incidence of cancer (Cr and As), nephrotoxicity and bone damage (Cd), and reduced reproductive function (Pb) (Neal and Guilarte, 2013; Brahmia et al., 2013; Uddh-Söderberg et al., 2015).

However, little is known about whether POPs (PCDD/Fs and PCBs) and heavy metals (As, Cd, Cr, and Pb) accumulate in the blood of captive pandas, and if they do, whether these pollutants present a health risk to these animals. To answer these questions, blood samples of Qinling pandas at SWARC were taken and analyzed for toxicants. We then examined relationships between toxicant concentrations and time spent in captivity, and panda health as assessed by hematological and biochemical parameters, and by analysis of sperm quality.

2. Materials and methods

All blood samples were collected from captive Qinling pandas housed in the Shaanxi Wild Animal Research Center ("SWARC": 34°06′N, 108°32′E). SWARC is located in Louguantai, Zhouzhi County, Xi'an city. It was established in 1987 and is the only center in the world for conservation of the Qinling subspecies of the giant panda.

Pandas were anesthetized with 25% ketamine at a dose of 8 mg/kg of panda body mass. Blood samples, which were residuals from physical examinations of the individual pandas at SWARC, were collected from the jugular vein of each of fifteen pandas ranging from 4 to 21 years of age that had been in captivity for 3–20 years. Blood samples were placed in EDTA tubes for hematological analysis of heavy metals, PCDD/Fs, and PCBs, and into serum tubes for biochemical analysis. Fresh blood samples were digested and analyzed using standard methods, usually within 1 h of collection.

2.1. Heavy metal analysis

500-mL blood samples were placed into Teflon bombs to which were added 5 mL of HNO_3 for digestion with a microwave system (CEM, Mars 6, CEM, USA). After digestion, samples were diluted to 50 mL with deionized water. Concentration of Cr was measured using the air-–acetylene flame method (AAS; ZEEnit 700P, Analytik, Jena, Germany) with electrically modulated deuterium–HCl background correction. The hydride-forming element As was measured using the HS55 Hydride System (AAS; ZEEnit 700P, Analytik, Jena, Germany). Concentrations of Cd and Pb were measured using a graphite furnace AAS coupled to a MPE 60 (ZEEnit 700 P, Analytik, Jena, Germany) graphite autosampler with two-field mode Zeeman effect background correction. Heavy metal concentrations are expressed as ng/g blood (Chen et, 2017).

2.2. Analysis of PCDDs, PCDFs, and PCBs

Blood samples (volume = 10 mL) were freeze-dried before being spiked with ^{13}C-labeled surrogate standards (Environmental Protection Agency [EPA] method 1613B and 1668A) and undergoing accelerated solvent extraction (ASE350; Thermo, MA, USA) with dichloromethane: hexane (1:1). After determining the lipid content of each sample, the extract was adjusted to 50 mL with hexane; 15 g of acid silica (30% w/w) (44 g H_2SO_4 (98%, GR; Sinopharm, China) + 100 g Silica gel) was added to remove lipids. The acid silica was stirred for 2 h and the extract was poured through 5 g of anhydrous sodium sulfate (Sigma-Aldrich; St. Louis, MO, USA). All the extracts were concentrated to 2 mL by rotary evaporation.

All solvents were purchased from Fisher Scientific (Fairlawn, NJ, USA). Silica gel was obtained from Merck (silica gel 60; Darmstadt, Germany). Basic alumina was obtained from Aldrich (Brockmann I, standard grade; Milwaukee, USA). Florisil was obtained from Riedel-de Haën (60–100 mesh ASTM; Seelze, Germany). Calibration standard solutions, [13]C-12-labeled surrogate standards, and [13]C-12-labeled injection standards were purchased from Wellington Laboratories

(Guelph, Canada).

PCBs, PCDDs, and PCDFs were analyzed at the POP laboratory of the Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences (RCEES-CAS, Beijing, China); all concentrations were corrected for lipid weight. Sample extraction, cleanup, and chemical analysis followed established methods with some modifications (Liu et al., 2006, 2008). 12 dioxin-like PCB congeners were quantified by an isotope dilution method using high-resolution gas chromatography coupled with high-resolution mass spectrometry (HRGC/HRMS) (Liu et al., 2006, 2008). Total organic carbon (TOC) concentration was analyzed on a TOC Analyzer (OI Analyzer; College Station, TX, USA). A 0.1-g sample was weighed and loaded into the combustion cup, which was packed with quartz wool. Prior to combustion, the samples were wetted with 5% phosphoric acid and heated to 250 °C for 1 min to purge inorganic carbon. The signal was detected by non-dispersed infrared (NDIR) detection when flashed at 900 °C for 6 min in the combustion chamber.

The quantification of 17 PCDD/Fs homologues was done using HRGC/HRMS on an Agilent 6890 gas chromatograph coupled with an Autospec Ultima mass spectrometer (Waters Micromass, Manchester, UK) operating in the EI (Electron Impact Ionization) mode at 35 eV; the trap current was 600 IA. The GC was equipped with a CTC PAL auto-sampler. One- or two-mL samples were injected in splitless mode (splitless time = 2 min for PCDD/Fs) in a DB-5MS fused silica capillary column (60 m for PCDD/Fs and PCBs) with helium as carrier gas at a constant flow rate of 1.2 mL/min. The oven temperature programs were as follows: for PCDD/Fs, start 150 °C held for 3 min, 150–230 °C at 20 °C min^{-1} held for 18 min, 230–235 °C at 5 °C min^{-1} held for 10 min, 235–320 °C at 4 °C min^{-1} held for 3 min; for PCBs, start 120 °C held for 1 min, 120–150 °C at 30 °C min^{-1}, 150–300 °C at 2.5 °C min^{-1} held for 1 min.

2.3. Quality control and quality assurance

All data were subject to quality control and quality assurance. All glassware was washed two times with distilled water, and then with dichloromethane after use. After washing, glassware was dried for 6 h at 400 °C in a muffle furnace. All performance criteria required for the analysis of PCBs and PCDD/Fs followed US EPA methods (1668A and 1613B).[13]C-labeled surrogated standards (1668A-LCS and 1613-LCS) were spiked in the sample for qualification and quantification, and ^{13}C-labeled injection standards (EPA 68A-IS and 1613-IS) were added for recovery calculation. The recoveries of the surrogate standards ranged from 76.7 ± 25.2% and 49.2 ± 13.6% for PCB sand PCDD/Fs, respectively, which met the requirements of US EPA methods 1668A and1613B. Limit of detection (LOD) in the sample was defined as a signal to noise (S/N) ratio = 3. The LOD values were in the range of 0.01–0.82 pg/g for PCBs and 0.04–8.40 pg/g for PCDD/Fs. Laboratory blanks were analyzed with samples quality control at set intervals, and there was no detection of target compounds in the blanks.

2.4. Hematological and biochemical analysis

Blood samples for serum were centrifuged at 2000 rpm, usually within 1 h of collection, and the serum was removed and stored at −20 °C. The hematology and serum biochemistry of samples were analyzed at the Shaanxi Sengong Hospital, Xi'an City. Counts or concentration of white blood cells (WBC: ± 0.1 × 10^9 /L), red blood cells (RBC: ± 0.5 × 10^{12} /L), hemoglobin (HGB: ± 2 g/L), platelets (PLT: ± 10 × 10^3 /μL) and lymphocytes (± 0.5% LYM) were determined using a Micro 60 Hematology Analyzer (Horiba ABX, Montpellier, France; guaranteed cv of measurements 0.5–5%).

Total protein (TP: ± 1 g/L), globulin (GLB: ± 0.5 g/L), albumin (ALB: ± 0.5 g/L), sodium (Na: ± 2.5 mmol/L), potassium (K: ± 0.025 mmol/L), chlorine (Cl: ± 1 mmol/L), calcium (Ca: ± 0.01 mmol/L), glucose (GLU: ± 0.025 mmol/L), alanine
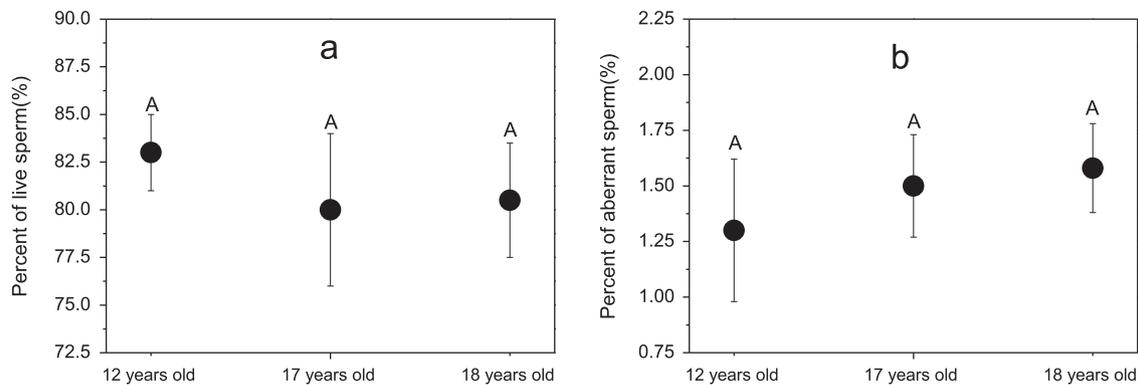
Fig. 1. The percent of live (a) and aberrant (b) sperm in samples from captive giant pandas of different ages. Data from different-age pandas were compared using one-way AVOVA. The data (mean ± SE, n = 3) with same letters among different ages are not significantly different (P > 0.05) based on Tukey's post-hoc test for multiple comparisons among means.

transaminase (ALT: ± 0.5 U/L), γ-Glutamyl transaminase (γ-GT: ± 0.1 U/L), aspartate transaminase (AST: ± 0.5 U/L), alkaline phosphatase (ALP: ± 2.5 U/L), lactate dehydrogenase (LDH: ± 5 U/L), blood urea nitrogen (BUN: ± 0.01 mmol/L), Creatine (CR: ± 0.5 μmol/L), creatine kinase (CK: ± 1 U/L), creatine kinase muscle B (CKMB: ± 1 U/L), hydroxybutyrate dehydrogenase (HBDH: ± 1 U/L), cholesterol (CHOL: ± 0.05 mmol/L), triglyceride (TRIG: ± 0.01 mmol/L), high-density lipoprotein cholesterol (HDL-C: ± 0.01 mmol/L), low-density lipoprotein cholesterol (LDL-C: ± 0.01 mmol/L), total bilirubin (TB: ± 0.01 mmol/L), and direct bilirubin (DB: ± 0.01 mmol/L) were analyzed using a Full Automatic Biochemical Analyzer 7080 (Hitachi Instrument Ltd., Tokyo, Japan; guaranteed cv of measurements < 5%).

2.5. Sperm analysis

Following anesthetization (as described above), sperm was taken from three wild panda (aged 12, 17 and 18 years; there were no significant differences in the percent of live or aberrant sperm among 12-, 17- and 18-year-old captive pandas; Fig. 1) that had been rescued from the Qinling Mountains between 2003 and 2013, and from nine captive pandas of corresponding ages. All of these sampled pandas were breeding at SWARC. Sperm samples were collected with an electro-ejaculator. A 20 × 1.9 cm probe delivering a current of 6–8 V was inserted into the rectum (Biojecktor 2001®, Comercial Varbó Ltda. Sáo Paulo-SP, Brazil) (Zhang and Wei, 2006). Semen samples were kept at 35 °C for immediate assessment of sperm motility and morphology (Gades and Matas, 2000).

2.6. Data analysis

All statistical analyses were done using the SPSS 20.0 software (IBM SPSS Statistics, 128 IBM Corp., USA Inc.); the significance level was set at α = 0.05.

3. Results

3.1. Blood levels of toxicants

Heavy metals and persistent organic compounds (POPs: PCDD/Fs and PCBs) were detected in all blood samples from all 15 individual pandas. Mean (± SE) concentrations of As, Cr, Pb, and Cd in the blood samples were 24.1 (3.05), 38.3 (2.64), 171.0 (11.60), and 2.4 (0.45) ng/g, respectively, and the concentrations of ΣPCDD/Fs and ΣPCBs were 2.52 (0.64) and 4.6 (0.58) ng/g lw. The means (SE) of WHO-TEQ (World Health Organization-Toxic Equivalent Quantity) of PCDD/Fs and PCBs in the blood samples were 0.28 (0.05) ng/g lw and 0.02 (0.02) ng/g lw, respectively (Fig. 2a, b and c). We identified out 14 and

12 congeners of PCDD/Fs and PCBs, respectively, in these samples. The dominant PCDD/Fs were OctaCDF and OctaCDD, which accounted for 53% and 20% of all congeners (Fig. 2d). For PCBs, the dominant congeners were PCB 105 and PCB 118, which accounted for 58% and 21% of all those detected (Fig. 2e).

Toxicant concentrations (As, Cd, Cr, Pb, ΣPCDD/Fs and ΣPCBs) in blood were significantly correlated with panda age and time in captivity (Table 1). The correlation coefficient and slope of the relationship between pollutant concentration and captivity time were greater than those of the relationship between pollutant concentration and age, suggesting that pollutants accumulated faster under captive conditions than would be expected in wild individuals. For heavy metals, the accumulation rate was greatest for Pb (Pb > As > Cd > Cr). For POPs, the accumulation rate for ΣPCDD/Fs was greater than that for ΣPCBs (Table 1).

3.2. Health assessment

Health assessments for the 15 pandas were based on the physical examination reports. There are no published data on these parameters for Qingling pandas, so the values were compared to published data for Sichuan pandas (Zhang and Wei, 2006). Threshold values of 27 hematological and biochemical parameters (red blood cell, white blood cell, hemoglobin, platelet, total protein, globulin, albumin, blood urea nitrogen, creatinine, sodium, potassium, chloride, calcium, glucose, alanine transaminase, aspartate transaminase, alkaline phosphatase, lactate dehydrogenase, creatine kinase, creatine kinase muscle B, hydroxybutyrate dehydrogenase, cholesterol, triglyceride, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, total bilirubin, and direct bilirubin) were used as criteria (Zhang and Wei, 2006). When compared to the range of values for healthy captive Sichuan pandas, the only parameter that was within the normal range for all 15 Qinling pandas was white blood cell count. Eleven of the measured health parameters for > 50% of the pandas studied were outside the normal range (Table 2): globulin (GLB), alanine transaminase (ALT), lactate dehydrogenase (LDH) and hydroxybutyrate dehydrogenase (HBDH) were higher than normal, whereas albumin (ALB), creatinine (CR), chloride (Cl), calcium (Ca), creatine kinase (CK), creatine kinase muscle B activity (CKMB), and total bilirubin (TB) were lower than normal.

Comparison of 22 blood biochemical and hematological parameters for captive Qinling pandas with values previously published for Sichuan pandas (Mainka et al., 1995) revealed no significant differences for 10 of 22 parameters (gray shading in Table 3). Rather, for all parameters except for red blood cell counts and creatinine, the mean values were greater for the Qinling subspecies than for the Sichuan subspecies, and significant differences between the two subspecies were detected for 12 of the 22 parameters (Table 3).
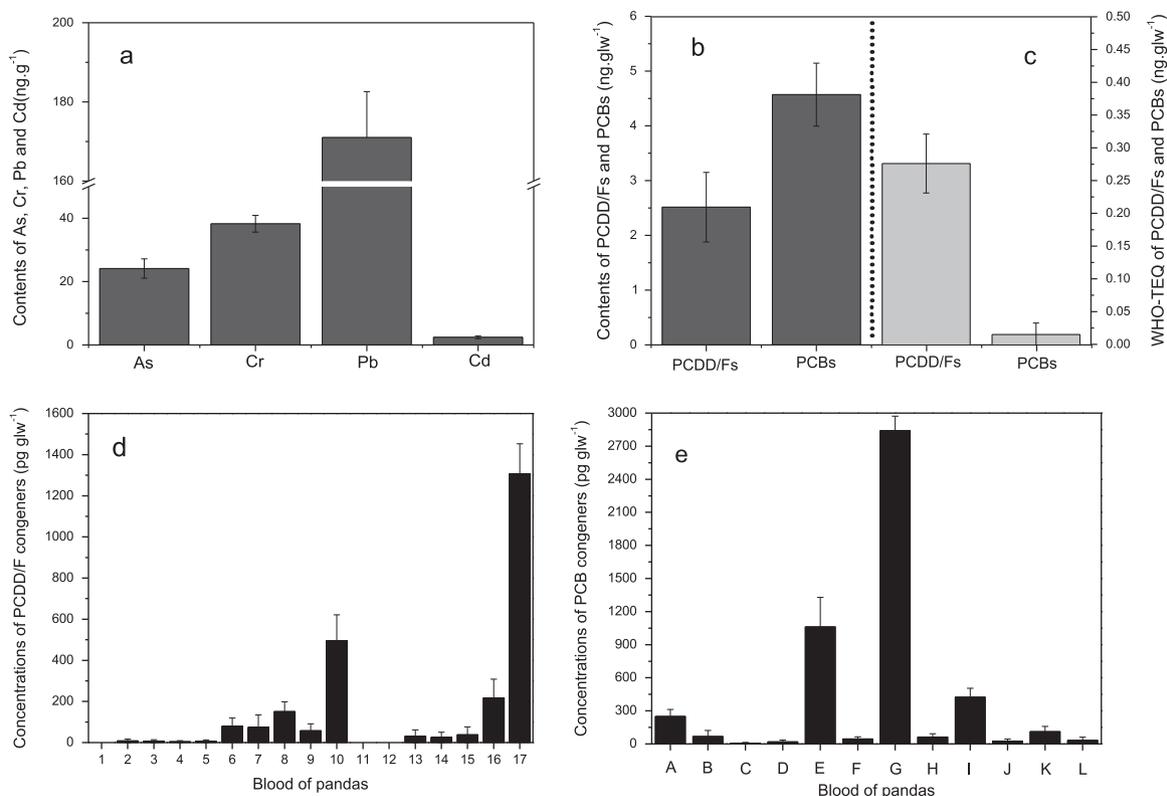
Fig. 2. The mean (± se) concentrations of heavy metals (As, Cr, Pb and Cd) (a), the concentrations of ΣPOPs (PCDD/Fs and PCBs) (b) and the mean (± se) WHO-TEQ of PCCD/Fs and PCBs (c) in the captive giant Qinling pandas blood (n = 15). The concentrations of PCDD/F (d) and PCB (e) congeners in the blood of captive pandas. Results are expressed as mean (± 1 SE of the mean) of 15 replicates. Numbers and letters on the x-axis of the panels denote different congeners of PCDD/F and PCBs. 1 = 2,3,7,8-TetraCDF; 2 = 1,2,3,7,8-PentaCDF; 3 = 2,3,4,7,8-PentaCDF; 4 = 1,2,3,4,7,8-HexaCDF; 5 = 1,2,3,6,7,8-HexaCDF; 6 = 2,3,4,6,7,8-HexaCDF; 7 = 1,2,3,7,8,9-HexaCDF;   8 = 1,2,3,4,6,7,8-HeptaCDF;   9 = 1,2,3,4,7,8,9-HeptaCDF;   10 = OctaCDF;   11 = 2,3,7,8-TetraCDD;   12 = 1,2,3,7,8-PentaCDD; 13 = 1,2,3,4,7,8-HexaCDD; 14 = 1,2,3,6,7,8-HexaCDD; 15 = 1,2,3,7,8,9-HexaCDD; 16 = 1,2,3,4,6,7,8-HeptaCDD; 17 = OctaCDD. A = PCB 77; B = PCB 81; C = PCB 126; D = PCB 169; E = PCB 105; F = PCB 114; G = PCB 118; H = PCB 123; I = PCB 156; J = PCB 157; K = PCB 167; L = PCB 189.

Table 1

Results of linear regression analysis of pollutant concentrations in panda blood with either age or time in captivity. Samples are from 15 captive pandas (Qinling subspecies) and the correlation coefficient (r) and slope (b) for each regression are presented along with the significant level (P) of r.

Pollutants	Panda age			Time in captivity		
	r	b	P	r	b	P
Arsenic	0.57	1.07	< 0.05	0.82	1.80	< 0.01
Cadmium	0.59	0.97	< 0.05	0.92	1.75	< 0.01
Chromium	0.67	0.19	< 0.05	0.94	0.31	< 0.01
Lead	0.71	5.11	< 0.01	0.82	6.92	< 0.01
ΣPCDD/Fs	0.77	0.32	< 0.05	0.92	0.45	< 0.01
ΣPCBs	0.69	0.24	< 0.05	0.73	0.30	< 0.05

The association between pollutant exposure and panda health was investigated using stepwise multiple regression to identify which toxicants were associated with changes in abnormal blood parameters. Five (ALT, LDH, Ca, Cl, TB) of the 11 parameters classified as abnormal were correlated with blood pollutant concentrations (Table 4). All pollutants except Cr were associated with abnormal blood parameters; Pb and ΣPCDD/Fs occurring most frequently in regression models.

3.3. Sperm quality

The high concentrations of As, Cr, Pb, Cd, ΣPCBs and ΣPCDD/Fs in captive panda blood may have some long-term negative effects. Long time behavioral observations at SWARC showed that the captive pandas had lower sexual desire than pandas of the same age captured from the

Table 2

Comparison of the hematological and biochemical parameters in the blood of 15 captive pandas (Qingling subspecies) with the normal range expected for healthy captive giant pandas (Zhang and Wei, 2006). Maximum and minimum values are presented for each parameter as well as the number of pandas for which a parameter for > 50% of the pandas is below (n < range) or above (n > range) the normal range.

Parameters	Min	Max	n < range	n > range	Normal Range
Globulin (g L^{-1})	19.0	69.4	1	10	28.0–39.0
Albumin (g L^{-1})	21.6	56.4	10	4	34.0–39.0
Creatinine (μmol L^{-1})	63.0	113.0	8	0	82.0–133
Chloride (mmol L^{-1})	86	116	8	1	96–103
Calcium (mmol L^{-1})	0.8	2.5	10	0	2.40–2.60
Alanine Transaminase (U L^{-1})	35.0	100.0	0	9	25.0–66.0
Lactate Dehydrogenase (U L^{-1})	770	1440	0	12	245–920
Creatine Kinase (U L^{-1})	35	437	13	0	254–1419
Creatine Kinase Muscle B (U L^{-1})	50	210	11	0	141–279
Hydroxybutyrate Dehydrogenase (U L^{-1})	774	1348	0	10	283–935
Total Bilirubin (μmol L^{-1})	0.8	12.7	10	4	5.10–6.10

wild. A comparison of sperm from wild and captive pandas found that the proportion of live sperm was significantly lower, and the aberrance ratio of sperm was significantly greater for captive pandas (Fig. 3), which could have been caused by high lead concentrations.

Table 3
Comparison of the blood biochemical and hematological parameters for captive Qinling subspecies and captive Sichuan subspecies. Data for Sichuan subspecies are from Mainka et al. (1995) and a Student's t-test was used to compare between the subspecies. Rows shaded in dark gray include those variables that did not differ between the subspecies (P > 0.05).

	Qinling Subspecies			Sichuan Subspecies			t-value	Sig.
	N	Mean	SD	N	Mean	SD		
WBC ($\times 10^9$ L^{-1})	15	6.4	1.8	16	7.0	2.8	0.76	P > 0.05
ALB (g L^{-1})	15	33.7	10.3	16	28.0	4.0	1.99	P > 0.05
Na (mmol L^{-1})	15	125	8.4	16	128	12.0	0.74	P > 0.05
Cl (mmol L^{-1})	15	97	6.4	16	94	12.0	0.94	P > 0.05
Ca (mmol L^{-1})	15	2.2	0.43	15	2.5	0.78	1.52	P > 0.05
GLU (mmol L^{-1})	15	4.8	1.11	14	5.1	1.27	0.68	P > 0.05
AST (U L^{-1})	15	63.3	21.4	16	62.0	24.0	0.17	P > 0.05
ALP (U L^{-1})	15	160	46	16	125	92	1.31	P > 0.05
CK (U L^{-1})	15	146	102.9	16	221	288.0	0.98	P > 0.05
γ-GT (U L^{-1})	15	12.5	8.48	15	11.8	6.97	0.26	P > 0.05
RBC (10^{12} L^{-1})	15	4.8	0.99	17	6.8	0.83	6.30	P < 0.01
LYM (%)	15	18.9	5.1	18	1.0	1.70	13.1	P < 0.01
TP (g L^{-1})	15	70	6.7	16	62	8.0	2.88	P < 0.05
BUN (mmol L^{-1})	15	4.4	1.18	16	2.5	1.33	4.23	P < 0.01
CR (μmol L^{-1})	15	84.6	14.38	16	141.4	35.36	5.93	P < 0.01
K (mmol L^{-1})	15	4.5	1.03	16	1.4	0.40	10.77	P < 0.01
ALT (U L^{-1})	15	66.5	20.7	16	50.0	20.0	2.24	P < 0.05
LDH (U L^{-1})	15	1030	164	13	530	335	4.89	P < 0.01
CHOL (mmol L^{-1})	15	6.4	1.91	13	4.9	0.75	2.71	P < 0.05
TRIG (mmol L^{-1})	15	1.7	0.57	11	0.6	0.26	6.77	P < 0.01
TB (μmol L^{-1})	15	5.0	3.57	14	2.5	1.28	2.46	P < 0.05
DB (μmol L^{-1})	15	4.8	3.40	13	1.4	1.23	3.62	P < 0.01

Abbreviation: RBC, Red Blood Cell; WBC, White Blood Cell; LYM, lymph cell; TP, Total Protein; ALB, albumin; BUN, Blood Urea Nitrogen; CR, Creatinine; Na, sodium; K, potassium; Cl, chlorie; Ca, calcium; GLU, glucose; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; CK, creatine kinase; γ-GT, γ-Glutamy transaminase; CHOL, cholesterol; TRIG, triglyceride; TB, total bilirubin; DB, direct bilirubin.

Table 4
Results of stepwise multiple regression of abnormal hematological and biochemical parameters in the blood of 15 captive pandas (Qingling subspecies) and six environmental pollutants (ΣPCDD/Fs, ΣPCBs, As, Cd, Cr and Pb). Pollutants that were positively and negatively associated with each parameter are listed along with the R^2 value for the regression model. There were no significant relationships between blood pollutant concentrations and concentrations of albumin, globulin, creatinine, creatine kinase, creatine kinase muscle B, or hydroxybutyrate dehydrogenase.

Parameters	Positive	Negative	R^2 (%)
Alanine transaminase (ALT)	ΣPCDD/Fs, Cd	None	65
Calcium (Ca)	None	Pb	77
Chloride (CL)	ΣPCBs	ΣPCDD/Fs	84
Lactate dehydrogenase (LDH)	As	Pb	59
Total bilirubin (TB)	ΣPCBs, ΣPCDD/Fs, Pb	None	85

4. Discussion

We previously showed that captive giant pandas are exposed to toxic chemicals via their food and that captive pandas are exposed to higher concentrations of pollutants than wild pandas (Chen et al., 2016). Heavy metals and persistent organic compounds (PCDD/Fs and PCBs) were detected in all blood samples of sampled Qinling pandas. The mean concentration of Pb in panda blood (171 ng/g) exceeds the safe level for humans by about 71% (U.S. Department of Health and Human Services, 1991), and is considerably higher than that reported for Grizzly bears (*Ursus arctos*, about 44 ng/g) or black bears (*Ursus americanus*, 16 ng/g) in Yellowstone National Park (Rogers et al., 2012). The mean concentrations of As, Cr, and Cd in the blood of captive pandas also were higher than those in human blood (but no comparable data are available for other Ursidae) (Wu et al., 2001; Ikeda et al., 2011). The mean concentration of ΣPCBs in panda blood was much lower than that reported for polar bears (*Ursus maritimus*) in East
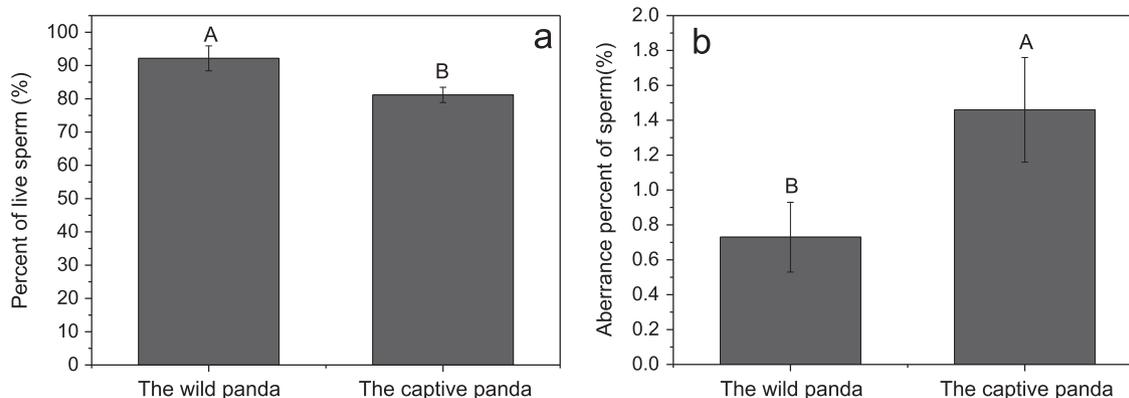
Fig. 3. Percent of live (a) and aberrant sperm (b) in wild and captive Qinling pandas. The data (means ± SE, n = 3 for the wild, and n = 9 for the captive) with different letters between the wild and captive pandas (a and b) are significantly different; P < 0.05, t-test. There were no significant different in percent of live sperm and percent of aberrant sperm among 12-, 17- and 18-year-old captive pandas (note see Fig. 1).

Greenland (3077 ng/g lw) (Gebbink et al., 2008) and the Southern Beaufort and Chukchi Seas (6026 ng/g lw) (Knott et al., 2012), probably because of the different diets of the two species. To our knowledge, blood PCDD/Fs concentrations have not been reported for other members of the Ursidae. Thus, we also compared our data with those reported for Chinese children living in urban areas. The mean concentration of PCDD/Fs in the blood of captive pandas was slightly higher than that in blood of children (0.21 ± 0.16 ng/g lw; Shen et al., 2010).

The means (SE) of WHO-TEQ of PCDD/Fs and PCBs in panda blood were 0.28 (0.05) ng/g lw and 0.02 (0.02) ng/g lw, respectively. Based on toxic equivalency factors (Berg et al., 2006), the congeners contributing the greatest toxicity in the blood of pandas were 2,3,4,7,8-PentaCDF, 1,2,3,4,7,8-HexaCDD and 1,2,3,7,8,9-HexaCDD. The dominant congeners in blood, which were OctaCDF, OctaCDD, 2,3,3′,4,4′-PentaCB and 2,3′,4,4′,5-PentaCB, were not the congeners with the greatest toxicity. Compared with published data, four congeners (1,2,3,7,8,9-HexaCDF, 1,2,3,4,7,8-HexaCDD, 1,2,3,6,7,8-HexaCDD, and 1,2,3,7,8,9-HexaCDD) detected in blood but not in droppings or food material (Chen et al., 2016) were detected in atmospheric deposition and likely were absorbed by the pandas when breathing contaminated air (Chen et al., 2017). These data indicated that those pollutants accumulated faster under captive conditions than under natural conditions, and suggests that the main source of pollutants for captive pandas are their food and contaminated air. Reducing toxic emissions and strictly controlling the quality of food provided to captive pandas should help protect and preserve captive panda populations.

Health assessments suggest that the health of captive Qinling pandas is compromised by pollutant exposure. More than 50% of the pandas studied had blood variables outside the normal range: globulin (GLB), alanine transaminase (ALT), lactate dehydrogenase (LDH), and hydroxybutyrate dehydrogenase (HBDH) were higher than normal, whereas albumin (ALB), creatinine (CR), chloride (Cl), calcium (Ca), creatine kinase (CK), creatine kinase muscle B activity (CKMB), and total bilirubin (TB) were lower than normal. The liver is a target site of many toxic chemicals, and high levels of LDH, ALT, and HBDH are associated with liver damage. ALT is a liver enzyme, which is released into the blood when liver cells have been damaged (Kim et al., 2004; Puoti et al., 2000). LDH is a marker of tissue breakdown and elevated levels of LDH and HBDH indicate liver lesions (Wang et al., 2006). The kidney is another target site of many toxic chemicals, and abnormal levels of ALB and CR indicate impaired kidney function (Chen, 2006). Environmental stress, including pollution, often results in the accumulation of reactive oxygen species (ROS), which can result in membrane damage. ROS are scavenged by antioxidants such as bilirubin (Stocker et al., 1987), whose synthesis is induced in response to oxidative stress (Ossola and Tomaro, 1998). Low total bilirubin (TB) levels may be a consequence of scavenging ROS. Low levels of Cl and Ca are indicative of poor nutrition, which may result from gastrointestinal dysfunction. Five (ALT, LDH, Ca, Cl, TB) of the 11 parameters classified as abnormal were correlated with blood pollutant concentrations by stepwise multiple regression analysis. This suggests that liver and kidney function of pandas may have been damaged by pollutants.

Our results provide the first direct evidence that blood of Qinling pandas contains significant concentrations of toxicants. Pb is particularly worrisome because its accumulation rate was greatest among the four assayed heavy metals and it can reduce reproductive function (Falcón et al., 2003). Long-term behavioral observations at SWARC have shown that captive pandas had lower sexual desire than pandas of the same age captured from the wild and we observed that proportion of live sperm was significantly lower in captive pandas; this result could be caused by high lead concentrations.

5. Conclusion

High concentrations of As, Cr, Pb, Cd, PCBs, and PCDD/Fs in captive

pandas are associated with negative indicators of panda health. Our results also highlight the severity of environmental issues associated with conservation of pandas in captivity. The critically small population size of Qingling pandas—less than 350 individuals remain— means that urgent action is required because this subspecies exposed to higher concentrations of POPs and heavy metals. Given that the primary source of toxic chemicals is poor air quality (Chen et al., 2017), which also results in contaminated food, a short-term solution to address this issue is to relocate breeding centers to less contaminated areas and to strictly control the quality of the food provided to captive pandas. A long-term and more sustainable solution will rely on improving air quality through reducing emissions of toxic chemicals.

References

Berg, M.V.D., Birnbaum, L.S., Denison, M., Vito, M.D., Farland, W., Feeley, M., 2006. The 2005 World health organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. Toxicol. Sci. 93, 223–241.

Brahmia, Z., Scheifler, R., Crini, N., Maas, S., Giraudoux, P., Benyacoub, S., 2013. Breeding performance of blue tits (*Cyanistes cauleus ultramarines*) in relation to lead pollution and nest failure rates in rural, intermediate, and urban sites in Algeria. Environ. Pollut. 174, 171–178.

Centers for Disease Control, 1991. Preventing lead poisoning in young children: a statement by the centers for disease control, October. (U.S. Department of Health and Human Services, 1991).

Chen, 2006. How to Analyse the Physical Examination Report. Jindun Press, Beijing, pp. 140–144 (in Chinese).

Chen, Y.P., Maltby, L., Liu, Q., Song, Y., Zheng, Y.J., Ellison, A.M., 2016. Captive pandas are at risk from environmental toxins. Front. Ecol. Environ. 14, 363–367.

Chen, Y.P., Zheng, Y.J., Liu, Q., Song, Y., An, Z.S., Ma, Q.Y., et al., 2017. Atmospheric deposition exposes pandas to toxic pollutants. Ecol. Appl. 27, 343–348.

Eqani, S.A., Cincinelli, A., Mehmood, A., Malik, R.N., Zhang, G., 2015. Occurrence, bioaccumulation and risk assessment of dioxin-like PCBs along the Chenab River, Pakistan. Environ. Pollut. 206, 688–695.

Falcón, M., Viñas, P., Luna, A., 2003. Placental lead and outcome of pregnancy. Toxicology 185, 59–66.

Fernandez-Rodriguez, M., Arrebola, J.P., Artachocordón, F., Amaya, E., Aragones, N., Llorca, J., 2015. Levels and predictors of persistent organic pollutants in an adult population from four Spanish regions. Sci. Total Environ. 538, 152–161.

Gades, J., Matas, C., 2000. Sperm factors related to in vitro penetration of porcine oocytes. Theriogenology 54, 1343–1357.

Gebbink, W.A., Sonne, C., Dietz, R., 2008. Tissue-specific congener composition of organohalogen and metabolite contaminants in East Greenland polar bears (Ursus maritimus). Environ. Pollut. 152, 621–629.

Gustavson, L., Ciesielski, T.M., Bytingsvik, J., Styrishave, B., Hansen, M., Lie, E., et al., 2015. Hydroxylated polychlorinated biphenyls decrease circulating steroids in female polar bears (Ursus maritimus). Environ. Res. 138 (1), 191–201.

Ikeda, M., Ohashi, F., Fukui, Y., Sakuragi, S., Moriguchi, J., 2011. Cadmium, chromium, lead, manganese and nickel concentrations in blood of women in non-polluted areas in Japan, as determined by inductively coupled plasma-sector field-mass spectrometry. Int. Arch. Occup. Environ. Health 84 (2), 139–150.

Kim, H.C., Nam, C.M., Sun, H.J., Han, K.H., Oh, D.K., Suh, I., 2004. Normal serum aminotransferase concentration and risk of mortality from liver diseases: prospective cohort study. Br. Med. J. 328, 983–986.

Knott, K.K., Boyd, D., Ylitalo, G.M., O'Hara, T.M., 2012. Lactational transfer of mercury and polychlorinated biphenyls in polar bears. Chemosphere 88, 395–402.

Li, Y.M., Jiang, G.B., Wang, Y.W., Wang, P., Zhang, Q.H., 2008. Concentrations, profiles and gas-particle partitioning of PCDD/Fs, PCBs and PBDEs in the ambient air of an E-waste dismantling area. Southeast China Chin. Sci. Bull. 53, 521–528.

Liu, H., Zhang, Q., Cai, Z., Li, A., Wang, Y., Jiang, G., 2006. Separation of polybrominated diphenyl ethers, polychlorinated biphenyls, polychlorinated dibenzo-p-dioxins and dibenzo-furans in environmental samples using silica gel and florisil fractionation chromatography. Anal. Chim. Acta 557, 314–320.

Lohmann, R., Breivik, K., Dachs, J., Muir, D., 2007. Global fate of POPs: current and future research directions. Environ. Pollut. 150, 150–165.

Mainka, S.A., He, T., Chen, M., Dierenfeld, E.S., 1995. Hematologic and serum biochemical values for healthy captive giant pandas (*Ailuropoda melanoleuca*) at the Wolong reserve, Sichuan, China. J. Zoo. Wildl. Med. 26, 377–381.

Neal, A.P., Guilarte, T.R., 2013. Mechanisms of lead and manganese neurotoxicity. Toxicol. Res. 2, 99–114.

Ossola, J.O., Tomaro, M.L., 1998. Heme oxygenase induction by UVA radiation. A response to oxidative stress in rat liver. Int. J. Biochem. Cell Boil. 30, 285–292.

Puoti, C., Castellacci, R., Montagnese, F., 2000. Hepatitis C virus carriers with persistently normal aminotransferase levels: healthy people or true patients? Dig. Liver Dis. 32, 634–643.

Qiu, J., 2013. Organic pollutants poison the roof of the world. Nature. http://dx.doi.org/10.1038/nature.2013.12776.

Rogers, T.A., Bedrosian, B., Graham, J., Foresman, K.R., 2012. Lead exposure in large carnivores in the Greater Yellowstone Ecosystem. J. Wildl. Manag. 76, 575–582.

SFA (State Forestry Administration), 2015. The 4th National Survey Report on Giant Panda in China. Science Press, Beijing in Chinese.

Sfriso, A., Facca, C., Raccanelli, S., 2014. PCDD/F and dioxin-like PCB bioaccumulation by Manila clam from polluted areas of Venice lagoon (Italy). Environ. Pollut. 184, 290–297.

Shen, H., Ding, G., Han, G., Wang, X., Xu, X., Han, J., et al., 2010. Distribution of PCDD/Fs, PCBs, PBDEs and organochlorine residues in children's blood from Zhejiang, China. Chemosphere 80 (2), 170–175.

Stocker, R., Yamamoto, Y., Mcdonagh, A.F., Glazer, A.N., Ames, B.N., 1987. Bilirum is an antioxidant of possible physiological importance. Science 235, 1043–1046.

Uddh-Söderberg, T.E., Gunnarsson, S.J., Hogmalm, K.J., Lindegård, M.I., Augustsson, A.L., 2015. An assessment of health risks associated with arsenic exposure via consumption of homegrown vegetables near contaminated glassworks sites. Sci. Total Environ. 536, 189–197.

Wang, L.N., Song, Y.R., Shi, J., 2006. The value of HBDH and LDH in clinic diagnoses of myocardial and liver damage. Clin. Med. China 22, 128–129 (in Chinese).

Wu, M.M., Chiou, H.Y., Wang, T.W., Hsueh, Y.M., Wang, I.H., Chen, C.J., et al., 2001. Association of blood arsenic levels with increased reactive oxidants and decreased antioxidant capacity in a human population of Northeastern Taiwan. Environ. Health Perspect. 109, 1101–1107.

Zhang, Z.H., Wei, F.W., 2006. Giant Panda Ex-situ Conservation: Theory and Practice. Science Press, Beijing (In Chinese).