



Supplementary Materials for

Exceptionally low daily energy expenditure in the bamboo-eating giant panda

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Published 10 July 2015, *Science* **349**, 171 (2015)
DOI: 10.1126/science.aab2413

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Declaration: All the experiments described herein were approved by the ethical review committee of the Chinese Academy of Sciences, Institute of Zoology, Beijing and the Ethical committee of Beijing Zoo.

Materials and Methods:

Doubly-labelled water

We measured the daily energy expenditure (DEE, kJ/day) using the doubly labelled water (DLW) technique (30). This method has been previously validated by comparison to indirect calorimetry in a range of mammals including humans (30). Prior to performing work in the field we performed a pilot study to assess the time taken for isotopes to reach equilibrium in body water. During the pilot study animals were weighed and a 1 ml blood sample was obtained from the cubital vein into a vacutainer to estimate the background isotope enrichments of ^2H and ^{18}O (ref 31: method D). Blood samples were immediately heat sealed into several 100 μL glass capillaries (Vitrex Ltd) which were stored at 4 °C. Afterwards, a known mass of DLW (639700 ppm ^{18}O , 352980 ppm ^2H) was administered (IV, 0.3g/100g body mass). Syringes were weighed before and after administration ($\pm 0.001\text{g}$, Sartorius balance) to calculate the mass of DLW injected. Blood samples were taken after 4, 6 and 8 hours of isotope equilibration. Animals were sampled again 24h, and at 6, 9, 12 and 15 days post dose, and these blood samples were taken as close as feasible to whole 24 h periods (32) to estimate isotope elimination rates. Taking samples over multiples of 24h periods

minimizes the substantial day to day variability in DEE (33, 34). Capillaries that contained the blood samples were then vacuum distilled (35) in China (Speakman Laboratory, IDGB, CAS, Beijing), and water from the resulting distillate exported to the UK (Speakman Laboratory, IBES, University of Aberdeen, UK) where it was used to produce CO₂ and H₂ (methods in ref 36 for CO₂ and ref 37 for H₂). The isotope ratios ¹⁸O: ¹⁶O and ²H: ¹H were analyzed using gas source isotope ratio mass spectrometry (Optima, Micromass IRMS and Isochrom μ G, Manchester, UK). Samples were run alongside three lab standards for each isotope (calibrated to International standards) to correct delta values to ppm.

To evaluate whether the isotopes had reached equilibration in body water we compared the isotope enrichments at 4, 6 and 8h post dosing with the back extrapolated intercept from the washout curve (Supplementary Material: S1, Table S1.1, S1.2, Fig. S1). In all cases the isotope enrichments were lower than the back extrapolated intercept, suggesting the isotopes had reached equilibration under 4h. We used this estimate as a guideline to hold animals that were subsequently captured in the wild. Typical washout curves in the captive animals showed that it took about 15 days for the isotopes to come back down to background levels. In the field therefore we aimed to recapture animals in a time window spanning 8-12 days (Supplementary Material: S1, Fig. S1). Isotope enrichments were converted to values of daily energy expenditure using equation 7.17 (30).

The field work was conducted in Foping Nature Reserve, in the Qinling

Mountains of Shaanxi, China (N33°32'-33°45', E107°40'-107°55'). We performed the study in three free-living pandas which had been previously (about 24 months before the current study) fitted with high frequency GPS/VHF radio-collars (GPS4400M, 7000M, Lotek Wireless Inc., Ontario, Canada) in the context of other studies (38). Two of the pandas were males aged about 9-10 and 13-15, the third was a female aged about 8-9. After a light anesthesia which was conducted by a qualified veterinarian, the panda was put in a net and weighed with a spring scale and then a 1 ml background blood sample was taken from the cubital vein. Then, a known mass of DLW was administered (IV) according to the body mass of each animal. Based on the results of the pilot study, the pandas were recaptured for blood samples taken 3.5 to 4 hours and 8 to 10 days after the dosing. Both the DLW administration and blood sampling procedures were identical to the pilot study in the captive animals. All the samples were stored at 4 °C in the field research station before shipping to laboratory for distillation and analysis.

Physical activity measurement

In captivity, we conducted direct observation on pandas by using all-occurrence sampling (39). Briefly, the panda was observed continuously for a period of 100 minutes and the behavior it is engaged in is recorded at the end of each minute during each observation day. The behaviors were then divided into four categories: eating, lying, sitting and other activity. Then, we calculated the time when pandas were active (eating and other activity) and inactive (lying and sitting). In the wild, we used the

GPS radio-collars to measure the activity level of pandas. The collar measures the activity in two axes using accelerometers (we chose Activity Measurement Mode 1). Axis X measures acceleration in forward/backward motions, axis Y measures sideways as well as rotary motion. In this mode, two average activity values (X, Y) were calculated and stored at five minute intervals. The units of measurement are counts which show the activity intensity of animals. The counts are zero when animals were completely inactive. We then calculated the daily time distribution when animals were inactive and active from these activity data. We also measured the movement speed when foraging by direct observation from a short distance (usually 5-10 meters) by measuring the distance between each site to the next one where the panda stopped and ate, and the mean movement speed over the whole year by using the GPS collar data (details see Zhang *et al.*, 2014, 40) on the collared pandas that were used in the DLW measurement in this study to show the relative intensity of activity.

Assimilation efficiency

We measured the assimilation efficiency of three captive pandas over a period of 7 days. During this period we weighed all the food provided to the animals immediately prior to it being placed in the pen. The animals were observed eating to confirm they ingested the foods. We collected and weighed all food discards and feces at the end of each day. Samples of food provided to the animals and samples of the discards were collected each day and were dried at 60 °C for 2 weeks to obtain their

water and dry matter contents. We measured the energy contents of the dry food items using bomb calorimetry (Parr bomb calorimeter). Energy assimilation efficiency was calculated from the estimated energy consumed (mass of each food item consumed multiplied by the energy content) and the calculated energy in the feces (fecal dry mass multiplied by fecal energy content) after Finley *et al.*, (2011) (7).

Net energy assimilation

At the end of almost every day, for a period of 11 months (January to November 2014) the feces produced by three individual pandas were collected and weighed (n total = 961 animal days). Using the known dry matter contents of the feces for these three individual pandas from the assimilation efficiency trial (above) we converted the wet fecal production to dry fecal production and then using the estimated individual assimilation efficiencies for the same three individuals converted the daily dry fecal production to a daily net energy assimilation. We simultaneously monitored the ambient shade and sun temperatures at a site 5 km from the zoo every 30 minutes and averaged the 48 daily readings to give a simultaneous ambient temperature measurement contemporary to the net energy assimilation values. NEA is not directly equivalent to daily energy expenditure because it does not account for the energy lost in urine. To estimate the urine energy content we calculated the total water turnover from the DLW estimates ($= k_d \cdot N_o$) and assumed that 25% of this was accounted for by respiratory water loss (30). The remaining unfractionated water loss comprises water in urine and water in feces. We estimated the water lost in feces from the dried feces

in the assimilation trial (above). Because these feces are collected at the end of each day some evaporation will have occurred particularly from the feces deposited early in the morning. Hence the calculated water in feces is a minimal estimate and the resultant estimated water in urine is a maximum. We collected urine from the captive pandas and dried it to evaluate the % of urine that comprises solids, and then measured the energy content of these solids using bomb calorimetry. Multiplying the maximal estimate of urine content by the proportion of urine that was solids and the energy content of these solids provided an estimate of the energy lost in urine on the DLW measurement days.

Thermal imaging

We measured the surface temperatures of giant pandas, zebra, Holstein dairy cattle and Dalmatian dogs using thermal imaging cameras (FLIR Ltd). We selected these animals because they all have contrasting black and white pelages under normal illumination. Images were captured from animals at two ambient temperatures (3 to 6 °C) and (9 to 13 °C) from a distance of about 3m trying where possible to get images of the lateral surface with the animals at 90° to the camera. Temperatures were calculated using an assumed surface emissivity value of 0.95, using the ‘box’ function in the FLIR analysis software suite.

Thyroid hormone measurement

We took 2 ml blood samples from each captive animal for which we had measured the energy expenditure, on two occasions (September and February) to

minimize seasonal effects. Blood samples were stored on ice for about 2 hours before centrifuging at 3000g for 10 minutes and the plasma were stored at -20 °C until measuring in the next day. Thyroid hormones (tri-iodothyronine, T3 and thyroxine, T4) were assayed in duplicate using radioimmunoassay kits from Beijing North Institute of Biological Technology. Intra- and inter-assay coefficient of variation was 4.1% and 5.5% for T3, and 4.4% and 6.3% for T4, respectively.

Genetics

We used a bioinformatics approach to screen for giant panda specific variations in the genes related to thyroid hormone synthesis and metabolism. From the KEGG (Kyoto Encyclopedia of Genes and Genomes) database, we selected a total of 182 genes related to the thyroid hormone signaling pathway (entry ID hsa04919), and the thyroid hormone synthesis pathway (hsa04918). Eight genomes (giant panda, five other Carnivores, mouse and human) were used to search for orthologous genes of the 182 human genes. Of the 8 genomes, 6 were available in USCS genome browser (hg19/GRCh37) (29) including the giant panda (*Ailuropoda melanoleuca*), ferret (*Mustela putorius furo*), dog (*Canis lupus familiaris*), cat (*Felis catus*), human (*Homo sapiens*) and mouse (*Mus musculus*). The other two, the tiger (*Panthera tigris*) and polar bear (*Ursus maritimus*) were downloaded from published papers (41, 42).

Starting from the 100 species multiple alignments from UCSC (43), we cut the exon parts of the 182 genes from the alignments, based on the protein coding sequence (CDS) of the human genes, from which the sequences of the 6 mammals in

the USCS database mentioned above were selected. Then the human exon sequences were used to search against the other two genomes respectively by standalone ncbi-blast-2.2.28 software. The hits from the two genomes (tiger and polar bear) were combined with other 6 genome sequences and aligned again with *Clustalo* (44) to get final alignments of all the exons of the CDS's. After manual check of these alignments, the giant panda specific variations were searched for in the alignment dataset to find out substitutions or indels which could cause in-frame premature stop codons within open reading frame.

Supplementary Text:

S1: Details of the doubly-labelled water measurements of captive and free-living giant pandas

Patterns of isotope equilibration were followed in 4 captive pandas (animals C1 and C2 were both adult males and animal C3 was an adult female. Animals C1 and C2 were less than 14 year old, and Animal C3 was 26 years old. Animal C4 was a juvenile female, 3 years old). The details are shown in Table S1.1 which for both isotopes shows the isotope enrichments above background in ppm the back extrapolated intercept time point 0 (time of injection) and samples collected at 4, 6 and 8 hours post dose. Below these estimates are the proportional estimates of the enrichments at time points 4, 6 and 8 hours relative to the intercept pool. Note that in all 4 individuals the enrichments at times 4, 6 and 8h are all lower than the intercept enrichment for both isotopes. Since the isotopes were administered IV the expectation is that the isotopes would first pervade the body water pool and then flood into the intra- and extra-cellular spaces (see Speakman *et al.*, 2001) (45). Hence prior to equilibration the expectation is that blood enrichments will exceed the intercept. Since in all cases they do not these data suggest that equilibration was reached some time prior to 4hours.

Table S1.1. Isotope enrichments of 4 captive pandas during the equilibration

process.

Isotope	Time point	C1	C2	C3	C4	Mean
Hydrogen	Intercept	69.5885	61.4547	50.1942	37.21456	
	4	65.22	56.4	48.525	36.95	
	6	65.23	55.71	47.925	35.075	
	8	62.25	58.12	47.155	34.32	
	4	0.93722	0.91775	0.96674	0.992891	0.95365
	6	0.93737	0.90652	0.95479	0.942508	0.9353
	8	0.89454	0.94574	0.93945	0.92222	0.92549
Oxygen	Intercept	129.97	113.25	93.5036	70.59081	
	4	122.5	105.9	91.3	71	
	6	122.05	103.9	89.75	67.7	
	8	115.65	105.8	87.85	66.5	
	4	0.94253	0.9351	0.97643	1.005797	0.96496
	6	0.93907	0.91744	0.95986	0.959048	0.94385
	8	0.88982	0.93421	0.93954	0.942049	0.92641

Table S1.2 The dilution space ratios for all 4 individuals at the different sampling time points. Dilution space ratios were in the expected range.

Table S1.2. Dilution space ratios in captive pandas during isotope equilibration.

	C1	C2	C3	C4	Mean
Intercept	1.03038	1.01666	1.02771	1.04648	1.03031
4	1.03622	1.03589	1.03801	1.06008	1.04255
6	1.03225	1.02891	1.03316	1.06484	1.03979
8	1.02494	1.00428	1.0278	1.06898	1.0315
Mean	1.03095	1.02143	1.03167	1.0601	

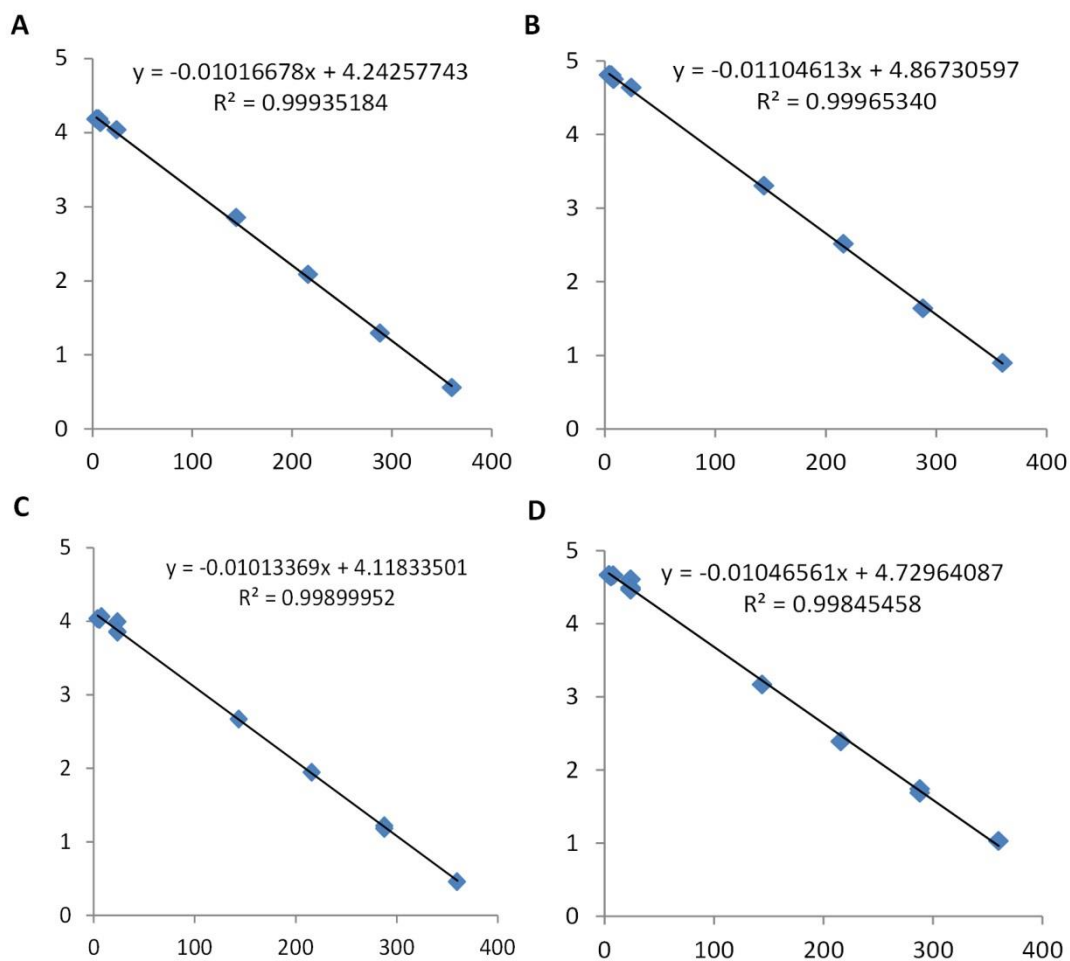


Fig. S1. Elimination curves for 2 individual giant pandas in captivity. We followed elimination of the isotopes sampling at 6, 9, 12 and 15 days post dose. **(A)** C1, Hydrogen. **(B)** C1, Oxygen. **(C)** C2, Hydrogen. **(D)** C2, Oxygen. The y-axis shows the logged isotope enrichment above background and the x-axis the time from dosing in hours. The fitted equations show the essential linearity of elimination ($r^2 > 0.998$) and the fitted elimination constants.

The elimination data in figure S1 emphasize the linearity of the elimination out to 15 days post dose. However, it is routine practice to eliminate final isotope enrichments

that are below 10ppm above pre-dose background and therefore to calculate the elimination constants to use in the analysis we truncated curves when the final values fell below 10ppm excess. Summary data for the DLW estimates of 5 captive individuals are summarized in Table S1.3. The animals C1-4 were the same as in the equilibration experiments. Animal C5 was also a 3 years old juvenile female. Measurements were made in October/November 2013 and April 2014. Estimated daily energy expenditure is in kJ/day using an RQ of 0.8. The mean elimination ratio (k_o/k_d) was only 1.0904 emphasizing the technical challenges of working with an animal that has such a low rate of metabolism.

Table S1.3. Summary data for DLW estimates in 5 captive giant pandas. k_d and k_o are the elimination constants for deuterium and oxygen18 respectively and the CO_2 production using equation 7.17 from Speakman (1997) (30) is shown in mols/hour and liters per day.

Individual	Kd	ko	ko/kd	BM(kg)	mols/h	L/day	kJ/day
C1	0.00984	0.01084	1.1014	108.5	1.8214	979.21	5875.2
C2	0.00998	0.01089	1.09094	117	1.75162	941.7	5650.0
C3	0.008616	0.009467	1.09869	97	1.36769	735.26	4411.6
C4	0.009697	0.010764	1.11003	79	1.73596	933.25	5599.5
C5	0.0069	0.00731	1.05867	54	0.42233	227.046	1362.3

Summary data for three individual pandas measured in the wild in April/May 2014 are

summarized in table S1.4 using the same measurement terms as in Table S1.3 for the captive individuals.

Table S1.4. Summary data for DLW estimates in three free-living giant pandas.

Individual	Kd	ko	ko/kd	BM(kg)	mols/h	L/day	kJ/day
W1	0.008553	0.009746	1.1395	71	1.3996	752.47	4514.8
W2	0.009447	0.010774	1.1405	80	1.7169	923.04	5538.2
W3	0.009415	0.010543	1.1199	127	2.6926	1447.6	8685.4

S2: Assimilation efficiency measurements

The dry matter and energy contents of the different components of the pandas' diet and feces are presented in table S2.1. Water content of the feces was related to the diet composition and was higher for shoot feces compared with leaf feces. The standard deviations and sample sizes are shown (SD, n). One panda (C2) produced distinctly different types of feces (leaf dominated and shoot dominated). The others only produced leaf dominated feces.

Table S2.1. Dry matter and energy contents of panda food items, feces and urine.

Item type	Dry matter	(SD, n)	Energy content	(SD, n)
	%		kJ/gram dry matter	
Fresh bamboo leaves	48.65	4.71, 4	18.34	0.32, 8
Bamboo leaves at end of day	75.15	5.93, 44	17.88	0.46, 8
Bamboo stems	62.43	3.30, 44	18.44	0.35, 8
Bamboo shoots	8.23	0.31, 2	18.44	0.18, 4
Apple	18.80	0.62, 2	15.82	0.05, 2
Egg	25.06	0.66, 2	26.65	0.08, 2
Carrot	10.87	0.62, 2	15.16	0.08, 2
Bread	56.26	0.68, 2	18.10	0.16, 2
Extruded food	95.84	0.42, 2	17.81	0.11, 4
Gruel	10.75	4.81, 64	18.96	1.31 11
Leaf dominated feces panda C2	27.44	1.25, 25	17.90	0.08, 2
Leaf dominated feces panda C4	31.32	1.88, 24	17.70	0.06, 2
Leaf dominated feces panda C5	31.07	1.93, 21	17.59	0.05, 2
Shoot dominated feces panda C2	12.19	2.18, 41	17.14	0.105, 4
Urine C1	2.23	0.22, 2	18.98	1.50, 2
Urine C2	2.85	0.21, 2	14.14	1.08, 2

Urine C3	2.44	0.22, 2	15.08	1.44, 2
Urine C4	3.16	0.19, 2	14.21	1.14, 2

We used these measurements, combined with the masses of the food presented to the animals each day and that left uneaten at the end of the day to calculate the energy budget of three pandas (animals C2, C4 and C5). The animals were measured for a total of 7 days. Data for day 1 was rejected in case the food intake the day before measurements started had been unusual and hence fecal production on day 1 might be abnormal, and the calculated energy intake and fecal energy production were summed over days 2 to 7. The budgets for these animals are presented in tables S2.2 to S2.4

Table S2.2. Energy budget for animal C2.

Food type	Dry mass ingested (g)	Energy intake (MJ)	% energy intake
Bamboo leaves	7813.3	143.29	50.6
Bamboo shoots	2337.5	43.10	15.2
Bread	0	0	0
Carrot	678.1	10.28	3.6
Extruded feed	3491.5	62.18	22.0
Apple	632.5	10.0	3.5
Gruel	312.0	5.91	2.1
Egg	308.6	8.22	2.9
TOTAL	15573.6	283.0	
TOTAL DAILY		47.16	
Feces type	Dry mass produced (g)	Energy in feces (MJ)	
Leaf dominated feces	10149.0	176.9	
Shoot dominated feces	2754.8	48.0	
TOTAL	12903.8	224.9	
Assimilation efficiency (%)		20.53	
Net energy assimilation (MJ/day)		9.68	

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Table S2.3. Energy budget for animal C4.

Food type	Dry mass ingested (g)	Energy intake (MJ)	% energy intake
Bamboo leaves	14262.8	261.58	85.8
Bamboo shoots	72.4	1.33	0.44
Bread	2055.2	37.20	12.2
Carrot	0	0	0
Extruded feed	0	0	0
Apple	0	0	0
Gruel	237.49	4.50	1.47
Egg	0	0	0
TOTAL		304.61	
TOTAL DAILY		50.77	
Feces type	Dry mass produced (g)	Energy in feces (MJ)	
Leaf dominated feces	15531.6	270.70	
Shoot dominated feces	0	0	
TOTAL	15531.6	270.7	
Assimilation efficiency (%)		11.13	
Net energy assimilation (MJ/day)		5.65	

Table S2.4. Energy budget for animal C5.

Food type	Dry mass ingested (g)	Energy intake (MJ)	% energy intake
Bamboo leaves	21973.5	403.0	91.8
Bamboo shoots	984.0	18.14	4.1
Bread	826.2	14.95	3.4
Carrot	0	0	0
Extruded feed	0	0	0
Apple	0	0	0
Gruel	154.0	2.92	0.66
Egg	0	0	0
TOTAL	15613.7	439.0	
TOTAL DAILY (MJ/day)		73.16	

Feces type	Dry mass produced (g)	Energy in feces (MJ)	
Leaf dominated feces	22335.5	389.3	
Shoot dominated feces	0	0	
TOTAL	22335.5	389.3	
Assimilation efficiency (%)		11.32	
Net energy assimilation (MJ/day)		8.28	

Calculated urine energy production

Water turnover across the five measured animals in captivity averaged 668 mls/hour (sd = 122). This is equivalent to 16.0 L per day. We assumed that 25% of this water loss was fractionated (i.e. respiratory water loss) following the standard assumption in the application of the DLW method (30). This meant that an estimated 12L per day was unfractionated turnover comprising the fecal and urinary water losses. We converted the daily weights of feces across animals C2, C4 and C5 into daily fecal water production using the calculated dry matter content of the feces (see table S2.1). There was a significant negative relationship between daily fecal water and ambient temperature (Fecal water (Liters/day) = 8.38 – 0.10 ambient temperature (°C): regression F = 400.8, p < 0.001, n = 961). Applying this equation for the ambient temperatures on the days the DLW technique was used yielded an estimated fecal water content of 7.0 L/day and hence the estimated urinary water production was 5.0 L/day. See methods section above for why this is a maximal estimate of urinary loss. Given the urinary solids comprised on average 2.64% of the wet urine, and the energy density of the urine solids was 15.68 kJ/g this gives an estimated maximal energy loss in urine of 2.1 MJ/day.

S3: Organ sizes in the giant panda

Table S3 details the masses of organs removed at autopsy from the giant panda. The data for the brain refer to a sample of 7 individuals averaging 60.8 kg in body mass (46). The data for the heart, liver, and kidney are from four individuals averaging 71.6 kg (47). In all cases we used the average body mass substituted into an allometric equation based on data across eutherian mammals to generate the expected sizes.

Table S3. Actual and expected organ sizes in the giant panda.

Organ	Size in panda (grams)	Expected size (grams)	Ratio	Prediction equation ref
Brain	252	288	0.875	Martin (1981) (48)
Heart	400	381.3	1.049	Stahl (1967) (49)
Liver	1243.7	1979	0.628	Prothero (1982) (50)
Kidney	237.1	319.1	0.745	Prothero (1984) (51)