Report

Current Biology

Diet Evolution and Habitat Contraction of Giant Pandas via Stable Isotope Analysis

Highlights

- We determine stable isotope values of ancient and modern pandas and sympatric species
- The isotopic trophic niches of ancient and modern pandas are distinctly different
- The niche widths of ancient pandas are about 3× larger than those of modern pandas
- The diet specialization of pandas was probably unfinished at the mid-Holocene

Authors

Han Han, Wei Wei, Yibo Hu, ...,
Yunbing Luo, Weicai Chen, Fuwen Wei

Correspondence

weifw@ioz.ac.cn

In Brief

Han et al. compare stable isotope ratios from the bones and teeth of extant and extinct pandas and show that their trophic niches are distinctly different, although both species had a C_3-dominated diet over time and space. The results indicate that ancient pandas possibly had more complex diets and habitats than do their modern counterparts.

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Current Biology
Report

Diet Evolution and Habitat Contraction of Giant Pandas via Stable Isotope Analysis

Han Han,[1,2,3] Wei Wei,[1,3] Yibo Hu,[1,4] Yonggang Nie,[1,4] Xueping Ji,[5] Li Yan,[1] Zejun Zhang,[3] Xiaoxue Shi,[6] Lifeng Zhu,[1] Yunbing Luo,[7] Weicai Chen,[8] and Fuwen Wei[1,2,3,4,9,*]
[1] Key Laboratory of Animal Ecology and Conservational Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China
[2] University of Chinese Academy of Sciences, Beijing 100049, China
[3] Key Laboratory of Southwest China Wildlife Resources Conservation, Ministry of Education, China West Normal University, Nanchong 637009, China
[4] Center for Excellence in Animal Evolution and Genetics, Chinese Academy of Sciences, Kunming 650223, China
[5] Yunnan Cultural Relics and Archaeology Institute, Kunming 650118, China
[6] Baoshan Museum, Baoshan 678000, China
[7] Hubei Provincial Institute of Cultural Relics and Archaeology, Wuhan 430077, China
[8] Natural History Museum of Guangxi Zhuang Autonomous Region, Nanning 530012, China
[9] Lead Contact
*Correspondence: weifw@ioz.ac.cn

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SUMMARY

The ancestral panda *Ailurarctos lufengensis*, excavated from the late Miocene, is thought to be carnivorous or omnivorous [1]. Today, giant pandas exclusively consume bamboo and have distinctive tooth, skull, and muscle characteristics adapted to a tough and fibrous bamboo diet during their long evolution [1, 2]. A special feature, the pseudo-thumb, has evolved to permit the precise and efficient grasping of bamboo [3, 4]. Unlike those of extant pandas, little is known about the diet and habitat preferences of extinct pandas. Prevailing studies suggest that the panda shifted to specialized bamboo feeding in the Pleistocene [5, 6]; however, this remains questionable. Pandas now survive in a fraction of their historical habitat [7], but no specific information has been reported. Stable isotope analyses can be used to understand diet- and habitat-related changes in animals [8]. Isotopic signals in bone collagen reflect dietary compositions of ancient human diets [9, 10] and dietary changes between historical and modern animal populations [11, 12]. Here, we conduct stable isotope analyses of bone and tooth samples from ancient and modern pandas and from sympatric fauna. We show that pandas have had a diet dominated by C_3 resources over time and space and that trophic niches of ancient and modern pandas are distinctly different. The isotopic trophic and ecological niche widths of ancient pandas are approximately three times larger than those of modern pandas, suggesting that ancient pandas possibly had more complex diets and habitats than do their modern counterparts. Our findings provide insight into the dietary evolution and habitat contraction of pandas.

RESULTS AND DISCUSSION

Today, giant pandas are represented by one iconic species, *Ailuropoda melanoleuca*, and they live only in the understory of particular mountains in southwestern China (Figure 1) [3]. We analyzed the bone collagen of modern pandas (collected between the 1970s and the 2000s) and of sympatric mammals from these mountains to determine the modern panda's trophic niche. $\delta^{13}C$ and $\delta^{15}N$ data from the modern panda and from sympatric mammal bone samples indicate three obvious trophic divisions of carnivores, herbivores, and giant pandas (Figure 2A; Table S1). Giant pandas had an average $\delta^{15}N$ value of −0.3‰ ± 0.9‰, 4.1‰ smaller than that of other herbivores (n = 78, p = 0.00). This indicates that modern pandas occupy a unique trophic niche in the context due to a highly specialized bamboo diet. The average $\delta^{13}C$ value of giant pandas is −23.5‰ ± 0.5‰, consistent with that of ground-dwelling forest feeders [13].

Isotopic analyses of ancient pandas and ancient sympatric fauna from two archaeological sites (Tangzigou and Xiaoshuijing) in northwestern Yunnan were used to determine the trophic niche of ancient pandas in the mid-Holocene (Table S2). These ancient samples were excavated simultaneously from the same geological layers with the same geologic age [14, 15]. Stable isotope analyses show that these animals segregate clearly in bivariate $\delta^{13}C$–$\delta^{15}N$ space according to food resources (Figure 2B; Table S2). None of the samples had high $\delta^{13}C$ values indicative of a diet rich in C_4 plants, presenting a similar forest landscape to the habitat of modern pandas. The nitrogen isotope composition of predators (mean: 8.5‰ ± 1.0‰, 7.3‰ to 9.2‰) is highly distinct from that of coeval plant-consumers (except pandas, mean: 5.5‰ ± 0.8‰, 3.6‰ to 6.6‰) in the region including Cervidae, Bovidae and Caprinae (n = 42, p = 0.00). Nevertheless, the average $\delta^{15}N$ values of these herbivores are statistically indistinguishable from those of sympatric ancient pandas (4.2‰ ± 1.3‰; n = 42, p = 0.18), suggesting that both have a parallel trophic niche in a mid-Holocene context. Although ancient pandas do have a slightly lower $\delta^{15}N$ value than do sympatric herbivores, these values are not as low as they are in modern pandas (Figure 2B). It is likely that isotopic

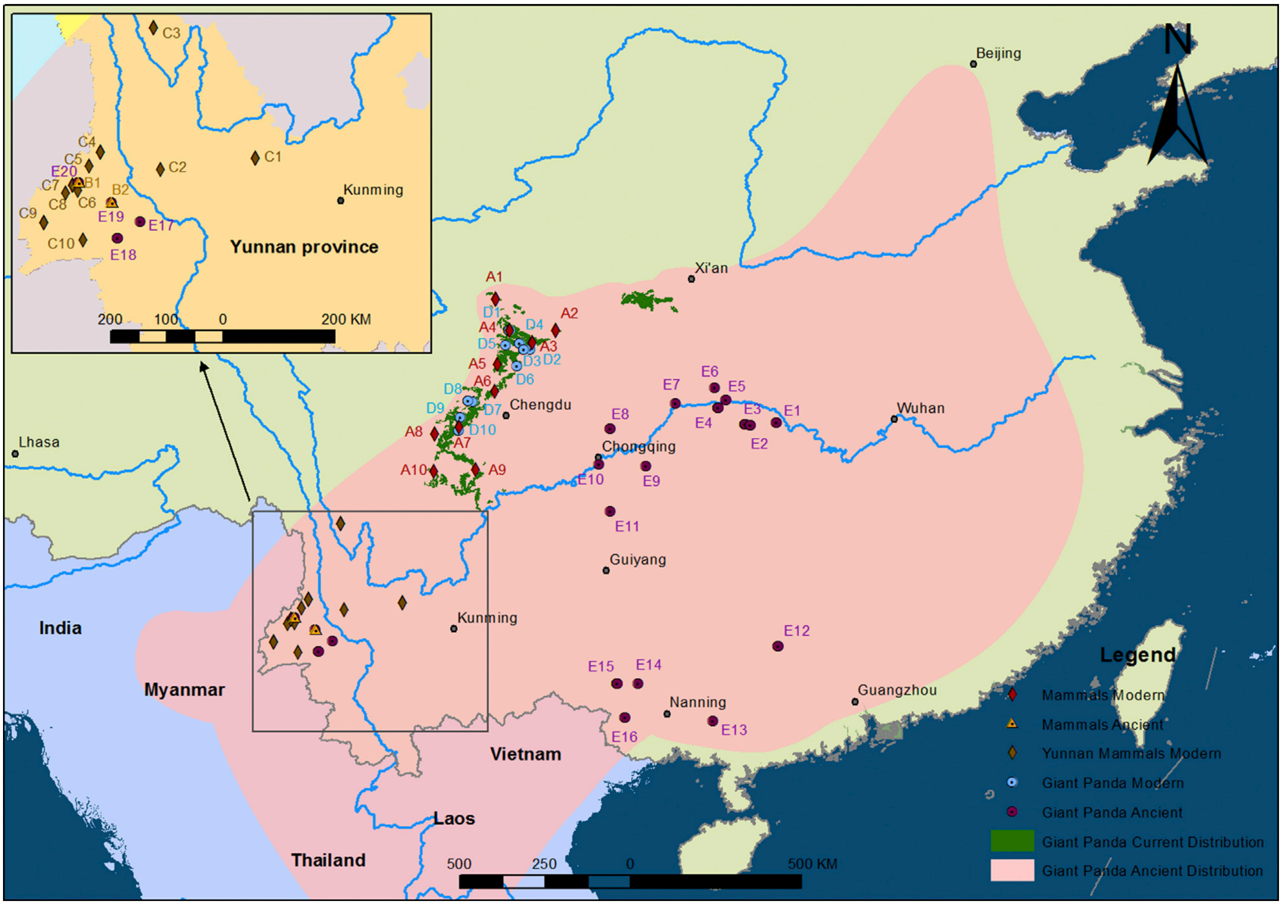

Figure 1. The Distribution of Ancient and Modern Giant Pandas and Sampling Sites in this Study

Ancient pandas (pink) occupied different habitats over southern, central, and northwestern China that extended as far north as Beijing and as far south as Myanmar, northern Vietnam, Laos, and Thailand. Modern pandas (green) only occupy forest habitats in Sichuan, Shaanxi, and Gansu provinces in China. The combinations of numbers and letters were used to represent all selected sample locations for pandas and sympatric fauna. A_1–A_X, modern mammal samples; B_1–B_X, ancient mammal samples; C_1–C_X, modern Yunnan mammal samples; D_1–D_X, modern giant panda samples; E_1–E_X, ancient giant panda samples.

variation may arise from climate changes and geochronologic differences or diet shifts. It is reported that the environmental conditions and climate features retained subtropical forests over thousands of years in our study area in northwestern Yunnan, and even the ecosystem type has not undergone much change and has remained stable for a long period of time [16, 17]. In order to explore whether there is isotopic variation in animals from different geological ages, we measured $\delta^{13}C$ and $\delta^{15}N$ isotopic values in modern animal bones around these two archaeological sites in northwestern Yunnan (Figure 2C; Table S3). Comparing these isotopic values with those from our ancient samples, we show that there are no statistically significant differences in $\delta^{15}N$ between modern and ancient carnivores (n = 22, p = 1.00) and herbivores (n = 72, p = 0.14). Furthermore, the habitat preferences of the mammal species presented at these sites clearly indicate that the area was covered with subtropical forest at the time the assemblage accumulated [6]. Therefore, temporal and climatic variations in isotopic values do not explain observed differences in $\delta^{15}N$ values or in trophic niches between modern and ancient pandas. These differences are most likely the result of different diets.

Records show that *A. microta* and later *A. baconi* (always found in abundance among Ailuropoda-Stegodon fauna during the Pleistocene) had a widespread distribution and occupied different habitats over southern, central, and northwestern China that extended as far north as Beijing and as far south as Myanmar, northern Vietnam, Laos, and Thailand (Figure 1) [3, 14, 15]. We measured bone collagen isotopes of 12 ancient pandas from seven archaeological sites in southern China (earlier than the late Neolithic Age) and compared them with collagen isotope patterns in modern giant pandas (Figure 3A; Table S4). $\delta^{13}C$ values ranged from $-25.8‰$ to $-22.3‰$ (mean: $-24.0‰ \pm 1.0‰$), and $\delta^{15}N$ values ranged from $2.7‰$ to $6.9‰$ (mean: $3.8‰ \pm 1.3‰$). According to distinctive diet-bone collagen isotopic discrimination factors in giant pandas [18], carbon isotopic analyses of all ancient pandas reveals that they had diets (mean: $-30.3‰ \pm 1.0‰$; range: $-32.1 \sim -28.6‰$) dominated by C_3 terrestrial food sources. Nonetheless, ancient and modern pandas are isotopically distinct ($\delta^{13}C$: n = 53, t = 7.93, p = 0.00; $\delta^{15}N$: n = 53, t = -12.53, p = 0.00). Ancient panda bone collagen is 4.1‰ enriched in $\delta^{15}N$ relative to that of modern pandas. The wider range of $\delta^{13}C$ and $\delta^{15}N$ values in ancient

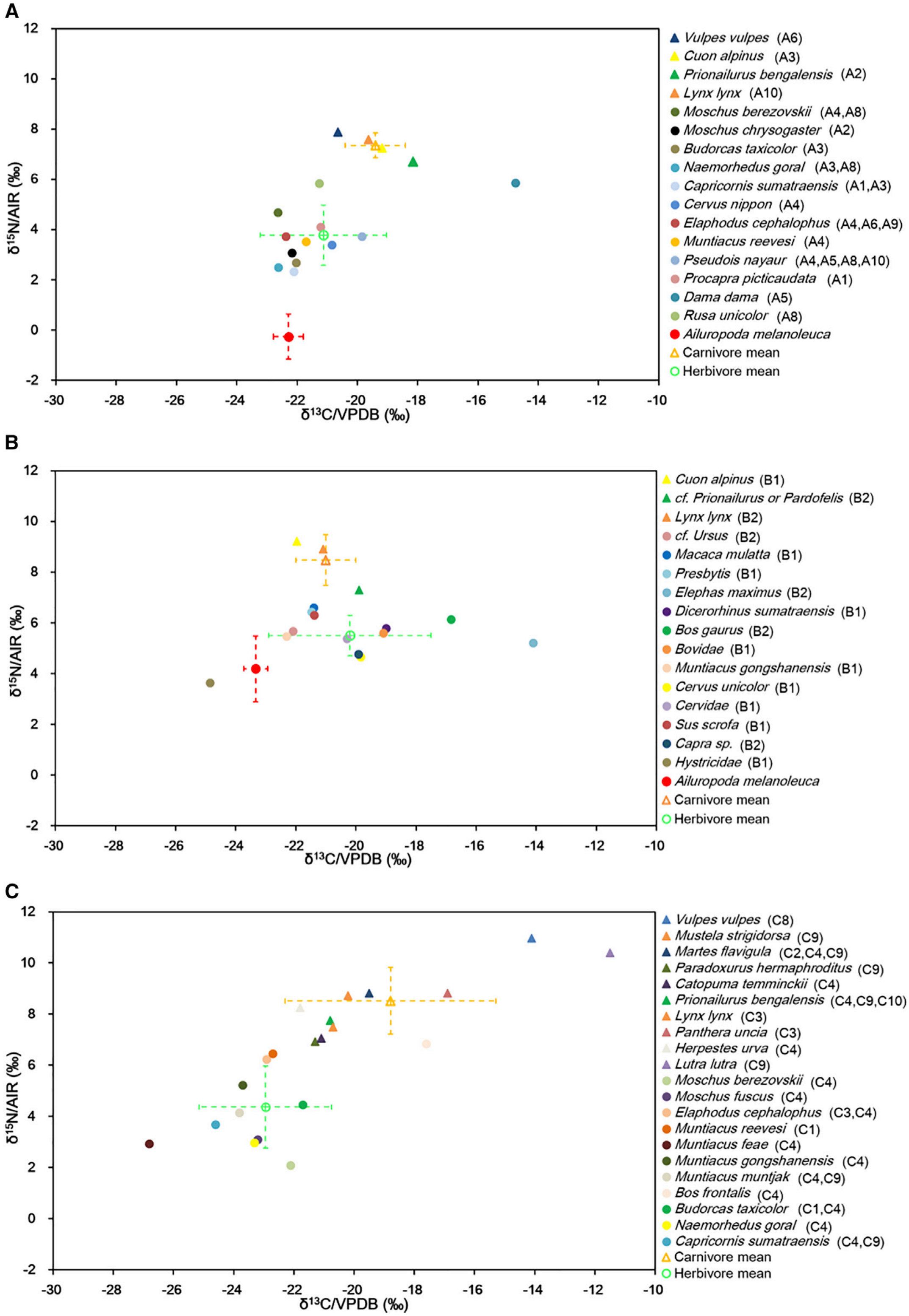

Figure 2. Pandas' and Other Species' Bone Collagen Stable Isotope Values for Ancient and Modern Samples

The trophic niche of giant pandas is decided by $\delta^{13}C$ and $\delta^{15}N$ values of bone collagen for (A) modern pandas with sympatric animals, (B) ancient Yunnan pandas with sympatric mammals, and (C) modern Yunnan mammals from the archaeological sites and their neighboring regions of (B). The solid triangles represent carnivores; the orange triangles and dashed lines represent the means and standard deviations (SDs) of carnivores, respectively; the green circles and dashed lines represent the means and SDs of herbivores, respectively. The red dots and dashed lines represent the means and SDs for pandas. See also Tables S1–S3.

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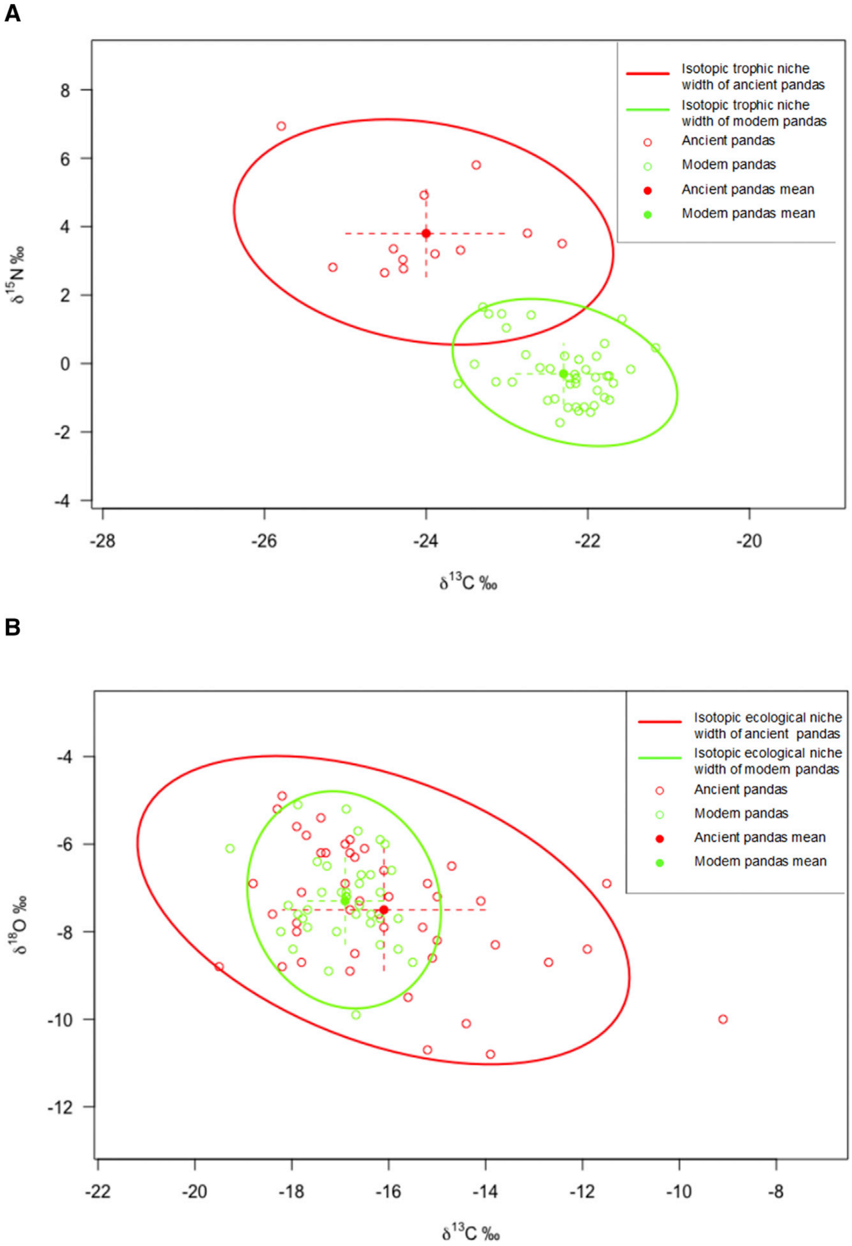

Figure 3. SEA$_B$ Representation of Isotopic Niche Width that Is a Bivariate Equivalent to SD in Univariate Analyses of Ancient and Modern Pandas

(A) δ^{13}C and δ^{15}N values of bone collagen from pandas are used to construct ellipses indicative of ancient (red) and modern (green) trophic niche widths.

(B) δ^{13}C and δ^{18}O values of tooth enamel from pandas are used to construct ellipses indicative of ancient (red) and modern (green) ecological niche widths. The dots and dashed lines represent the means and standard deviations (SDs) of ancient (red) and modern (green) pandas.

See also Table S4–S6.

pandas demonstrates that they may have had a more variable diet and hence were probably not exclusive bamboo feeders. It is possible that ancient pandas consumed a greater number of non-bamboo food sources than do contemporary pandas.

Because of the poor resistant ability of bone collagen to post-mortem diagenesis, tooth-enamel isotopes are a better choice for determining dietary shifts and ecological implications over long periods of time [19], and they have been used to investigate dietary variability, feeding changes, climatic features, and habitat types in human and non-human animal evolution [20–23]. We analyzed tooth isotopes to look at trophic and ecological niches of modern and ancient pandas, including *A. microta*, *A. baconi*, and *A. melanoleuca*, for much longer periods, spanning from the late Pliocene to mid-Holocene. Carbon and oxygen stable isotope ratios of ancient pandas (δ^{13}C: −16.1 ± 2.1‰, −19.5‰ ~ −9.1‰; δ^{18}O: −7.5‰ ± 1.4‰, −10.8‰ ~ –4.9‰) at archaeological sites show a wide range and also plot together with those of modern pandas (revised δ^{13}C: −16.9‰ ± 0.8‰, −19.3‰ ~ –15.5‰; δ^{18}O: −7.3‰ ± 1.0‰; −9.9‰ ~ –5.1‰) (Figure 3B; Tables S5 and S6). This suggests that ancient pandas might have had greater ecological flexibility than modern pandas have and that ancient pandas inhabited more environmental types, coinciding with most archaeological records from southern to northern China [15]. All pandas had isotope values consistent with pure C$_3$ diets mainly consisting of plants growing in a closed-canopy forest, supporting the fact that the dietary shift in pandas from carnivorous or omnivorous to vegetarian might have been accomplished by the end of the Pliocene. ^{13}C/^{12}C ratios provide evidence of an animal's diet and habitat conditions, while ^{18}O/^{16}O values are used to establish paleoclimatic proxies [23, 24]. In general, animals in cold and dry conditions have more positive δ^{18}O values than do those that inhabit wet and warm conditions [22]. The highly variable δ^{18}O values of ancient pandas suggest that they may have survived in a hot and moist (e.g., subtropical) area, which differs from the habitats of extant species. This is supported by fossil records excavated in southern China [15]. Given the much wider range of δ^{13}C and δ^{18}O values in ancient pandas, we speculate that they may have lived in a more variable environment, such as subtropical zones, forest fringes, or a connected zone of dense forest and open land.

To further delegate the trophic and ecological niche widths of ancient and modern pandas, we calculated SEA$_B$ and a corrected value for SEA$_C$ using collagen δ^{13}C and δ^{15}N and enamel δ^{13}C and δ^{18}O values. The results show a significant decrease in trophic niche width from SEA$_C$ = 4.4‰^{2} (SEA$_B$ = 4.4‰^{2}) for ancient pandas to SEA$_C$ = 1.5‰^{2} (SEA$_B$ = 1.5‰^{2}) for modern pandas (Figure 3A; Tables S1 and S4). The overlap areas between the two groups indicate that both ancient and modern pandas have the same food catalogs, such as bamboo. The SEA_C based on isotopic values in modern panda teeth is $2.7‰^2$ ($SEA_B = 2.5‰^2$) and is significantly narrower than that of ancient pandas ($SEA_C = 8.6‰^2$, $SEA_B = 8.3‰^2$; Figure 3B; Tables S5 and S6). It is interesting to note that the modern ellipse of pandas is totally covered by the ancient one, implying that the habitat of modern pandas is but a small part of the range of habitat types inhabited by ancient pandas. According to SIBER model analysis, the isotopic trophic and ecological niche widths of ancient pandas are approximately three times larger than those of modern pandas. This suggests that ancient pandas might have a more complicated diet not comprising exclusively bamboo, and they probably adapted to a variety of habitat types other than the cool forests of modern pandas.

Examining trophic and ecological niche widths via extinct and extant populations provides direct evidence of dietary specialization and habitat contraction in giant pandas and improves our understanding of their adaptation to modern environments. Although mid-Holocene pandas occupied a similar trophic level to that of sympatric herbivores, modern pandas have unique $\delta^{13}C$ and $\delta^{15}N$ values and a much lower trophic level. Paleontological and molecular evidence suggest that pandas switched to bamboo feeding ca. 2 million years ago [5, 6]; however, the first description of their bamboo diet is only a few hundred years old [14]. Contrary to earlier speculation, we show the panda was most likely not a specialized bamboo feeder until the mid-Holocene and that dietary components prior to this were complex. Pandas concentrated on the exploitation of C_3-dominated forest environments over time (starting from the late Pliocene) and space (such as Guangxi, Hunan, Hubei, Sichuan, Chongqing, Guizhou, and Yunnan) and had a subsistence strategy in more diverse climates and woody cover habitats historically.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes six tables and can be found with this article online at https://doi.org/10.1016/j.cub.2018.12.051.

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AUTHOR CONTRIBUTIONS

F.W.W. designed the research; H.H. and W.W. conducted the research. H.H., Y.G.N., X.P.J., X.X.S., L.Y., L.F.Z., Y.B.L., and W.C.C. collected the samples. F.W.W., H.H., W.W., and Y.B.H. wrote the paper with contributions from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and Algorithms		
SPSS 21.0	SPSS Inc.	https://www.ibm.com/analytics/spss-statistics-software
"SIBER" R package	Authored by Andrew Jackson and Andrew Parnell	https://cran.r-project.org/web/packages/SIBER/index.html
Other		
Stable isotope data	This paper	Available in Supplemental Information

CONTACT FOR REAGENT AND RESOURCE SHARING

Requests for further information should be directed to and will be fulfilled by the Lead Contact, Fuwen Wei (weifw@ioz.ac.cn).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The panda's trophic niche in modern habitat was determined by specimens from five mountains of Sichuan where most giant pandas inhabit now, along with sympatric herbivores and carnivores. Bone samples from 82 specimens with known wild origin were measured (Table S1). Specimens were labeled with information including their geographic origin, collection time and collector. These samples were supplied by China West Normal University, Wanglang Nature Reserve and Institute of Zoology, Chinese Academy of Sciences. The ancient panda's trophic niche was measured by sympatric faunal samples excavated simultaneously with the ancient panda samples from two archeological sites (Tangzigou and Xiaoshuijing) in northwestern Yunnan. These areas have always been the subtropical forests over thousands of years with relatively stable environmental conditions and climate features [16, 17]. These samples were conserved in Baoshan Museum and Yunnan Institute of Cultural Relics and Archaeology. Besides, we compared modern mammals from Kunming Institute of Zoology, Chinese Academy of Sciences deriving from these two archaeological and neighboring regions. The rest ancient pandas' teeth and bone samples were collected from China West Normal University, Natural History Museum of Guangxi Zhuang Autonomous Region, Chongqing Natural History Museum, Hubei Institute of Cultural Relics and Archaeology, Chengdu University of Technology.

METHOD DETAILS

Sample Collection

For modern specimens we only selected samples over 3-years-old as mothers' milk affects isotopic results [25]. For cases lacking age information we used samples that were clearly adult animals based on body size. All ancient giant panda samples (including teeth and bones) were collected from the panda's ancient distribution area including Yunnan, Sichuan, Hubei, Chongqing and Guangxi (estimated to be greater than the late Neolithic Age, Tables S2 and S6). All the ancient panda bones are identified to be adults by skeleton when excavated or damage degree of teeth or skull size if the skull was available [16, 17, 26, 27]. Most ancient (fossil and subfossil) fauna were dated based on archaeological context (e.g., presence within Pleistocene deposits). Some subfossil bones from northwest Yunnan were radiocarbon dated to 8740 ± 45 BP, 6895 ± 225 BP and 5025 ± 35 BP [16, 28].

Laboratory Treatment

Approximately 10 mg of tooth enamel powder was drilled from fragmented fossil teeth. Broken tooth surfaces were sampled as it is easier to separate enamel from dentine, meanwhile the morphology was not damaged. Powders were treated with 0.1 M acetic acid at room temperature for 30 min to remove secondary carbonates, and then rinsed many times with ultrapure distilled water to neutrality and dried overnight at 60°C [29]. This process led to a sample loss expected from the procedure. To analyze carbon and nitrogen isotope composition of bone samples, we extracted collagen from crushed bone samples, including modern and fossil, using a modified Longin method with the addition of an ultrafiltration step [30]. Samples were first demineralized in 0.5 M HCl for a couple of days at 4°C, with acid changes every few days. After no further bubbles were produced, samples were rinsed with deionized water and gelatinized for 48 h at 75°C in a pH 3 solution. The supernatant first went through a 0.45 μm filter, subsequently poured into ultrafilters to remove molecules less than 30 kDa. The purified solution was frozen and then lyophilized to generate the final collagen product.

Stable Isotope Analysis

Carbon and nitrogen isotope ratios of bone samples were simultaneously analyzed by a Flash Elemental Analyzer 1112 coupled with a Finnegan MAT253 isotope ratio mass spectrometer at the Institute of Geographic Sciences and Natural Resources Research,

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Chinese Academy of Sciences (CAS). The international references IAEA-600 was used to normalize N_2 and CO_2 reference gas in steel bottles. The analytical precision was 0.2‰ for $\delta^{13}C$ and $\delta^{15}N$ from the international reference materials (IAEA-600). C/N ratios of collagen were determined from the elemental analyzer. All subfossil bone samples measured have an average C/N ratio (3.2) in the range of 2.8–3.6, akin to those in modern bones (2.9–3.6 C/N ratio) [31], representing uncontaminated samples. Carbon and oxygen isotope ratios of teeth enamel were determined using GasBench II coupled to a Finnegan MAT253 isotope ratio mass spectrometer at the stable isotope laboratory, Institute of Geology and Geophysics, CAS. Data were normalized to CO_2 reference gas calibrated to NBS-19. The analytical precision was 0.15‰ for $\delta^{13}C$ and 0.20‰ for $\delta^{18}O$ through repeated analyses of the international reference materials (NBS19) and internal laboratory standard. Values were reported using the conventional permil (‰) notation where $\delta X = (R_{sample}/R_{standard} - 1) \times 1000$, ($\delta X$ represents $\delta^{13}C$, $\delta^{15}N$ or $\delta^{18}O$), by international isotope standards VPDB (Vienna Pee Dee Belemnite) for $\delta^{13}C$ and $\delta^{18}O$, and AIR (atmospheric air) for $\delta^{15}N$.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical Analysis

All datasets were examined to be normal distributed by Kolmogorov-Smirnov test. We conducted independent-samples t test to examine differences of stable isotope measurements between ancient and modern pandas. As there is some difference in sample size between each group, ANOVA with Bonferroni was used to explore pairwise differences if control variables exceed three including modern pandas, sympatric carnivores, herbivores and ancient pandas, sympatric carnivores, herbivores from Yunnan northwestern archaeological sites. Means and standard deviations (±SD) of the results were recorded. The significance level was set at $p \leq 0.05$. All statistical analyses were conducted using SPSS 21.0 (SPSS Inc., Chicago, USA). Carbon dioxide now in the atmosphere depleted in ^{13}C relative to preindustrial values, termed the "Suess effect" due to the burning of fossil fuels [32]. The $\delta^{13}C$ value of global CO_2 decreased from -6.3‰ in preindustrial times to -7.8‰ by the end of the 20th century [33]. For comparative purposes, modern samples have had their $\delta^{13}C$ values adjusted to recent atmospheric changes based on ice core records, depending on the estimated time samples collected [29].

Isotopic Niche Width

The multivariate analysis of variance was performed to determine and compare isotopic niche width of modern and ancient giant pandas. Isotopic niche width was estimated using a Bayesian approach based on multivariate, standard ellipse-based metrics, since this method is quite appropriate for comparisons between small and different sample sizes and identifies the niche width differences between most members in the population. Trophic niche widths were defined and drawn in bivariate plots using stable isotope $\delta^{13}C$ and $\delta^{15}N$ values decided from bone collagen, meanwhile ecological niche metrics were evaluated by isotope composition of tooth enamel ($\delta^{13}C$ and $\delta^{18}O$) [34]. The analysis was implemented in the R package SIBER [35] to generate standard ellipse areas (SEA_B): a bivariate equivalent to the standard deviation and a corrected measure for small sample sizes (SEA_C) [36]. The area within an ellipse is calculated by a subsample (40%) of bivariate data, in two cases, the ratios of nitrogen $^{15}N/^{14}N$ and carbon $^{13}C/^{12}C$ or oxygen $^{18}O/^{16}O$ and carbon $^{13}C/^{12}C$, that best explain the covariance, and by resampling multiple times estimates an error term associated with this value. The statistical significance of differences in SEA_C between sample sets was based on the proportional outcome of 10^6 repeats [36]. Finally, using the pairwise niche comparison approach based on SIBER, modern and ancient ellipses were calculated, allowing isotopic trophic and ecological niche width comparisons.

DATA AND SOFTWARE AVAILABILITY

The data were detailed in Tables S1–S6. The software required to perform the analyses described in the Quantification and Statistical Analysis section is available in the SPSS 21.0 (SPSS Inc., Chicago, USA) and open source R v3.0.3 [35].