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Metabolomic Studies of Urinary Metabolic Profiling in Sichuan Golden Monkey, *Rhinopithecus roxellana*

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Abstract.- Chinese snub-nosed monkeys (genus *Rhinopithecus*, subfamily Colobinae) include three species, *R. bieti*, *R. brelichi* and *R. roxellana*. They represent physiological strategies of adaptation to the environmental stresses. In this study, we investigated urinary metabolomics by GC-MS metabolic profiling approach, to correlate physiological states and metabolic responses of Sichuan golden monkeys to seasonal variations based on gender differences. Multivariate statistical analysis was used to process the integrated dataset generated from the GC-MS. A total of sixty-one endogenous metabolites were identified in the urine of Sichuan golden monkeys, and six and ten endogenous components were identified to be associated to metabolic responses to gender differences and seasonal variations, respectively. The results provide a preliminary understanding of the metabolic mechanisms of Sichuan golden monkeys. A number of metabolic changes indicate underlying adaptation and survival of these species to extraneous environmental factors, which may help improving conservation management strategies.

Keywords: Metabolic profiling, gender differences, seasonal variations, Sichuan golden monkey

INTRODUCTION

The animal organism is a complex and dynamic array that involves many interacting metabolic pathways. Tight control over the metabolic profile of an individual, referred to as homeostasis, is required to maintain health. However, the environment has an important role in the natural selection of organisms. Environment changes or climatic fluctuations can intrigue organisms to evolve rapidly into different morphologic or taxonomic groups, or create new functions specialized in different individuals living environments (Huntley and Webb, 1989). Animals native to high altitudes, and surviving over thousands of years in the highlands, have developed various behavioral and physiological strategies to deal with the environmental challenges (Wang *et al.*, 2010).

Metabolomics, an emerging field concerned with the study of an organism's physiological state at the substrate level, offers a means to obtain the dynamic multiparametric responses of a living system to various intrinsic and extrinsic parameters including gender, age, genetics, environment, drugs, diet and microbiome modulations (Nicholson *et al.*, 1999; Collino *et al.*, 2009). Biofluids, such as urine has been heavily used to explore the systematic modification of the metabolome due to its ease of collection, repeated samples, rich metabolite composition and, especially, the non-invasivity of urine sample collection (Adamko *et al.*, 2007; Beckonert *et al.*, 2007). The endogenous metabolites that are typically profiled include organic acids, amino acids, amines, sugars, steroids, nucleic acid bases, and other substances, which are intermediates in cellular metabolism (Pasikanti *et al.*, 2008). As these small molecular metabolites vary greatly in terms of their physicochemical properties and acid-base characteristics, several high-throughput analytical tools have been investigated to resolve these analyses, including NMR (^{1}H nuclear magnetic resonance), LC-MS (liquid chromatography-mass spectrometry), GC-MS (gas chromatography-mass spectrometry) and UPLC-MS (Ultra performance liquid chromatography) (Collino *et al.*, 2009). Among the tandem techniques investigated, the GC-MS technique has proven to be a potentially useful method, based on its high sensitivity, peak resolution and reproducibility (Pasikanti *et al.*, 2008). Recent metabolic profiling technology has utilized multivariate statistics to extract meaningful biological information from the resultant complex and huge data sets (Schlotterbeck *et al.*, 2006; Collino *et al.*, 2009). Metabolomics has been used for physiological studies of animal

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organisms. For example, Robert *et al.* (2008) reported that a number of metabolic changes in the Antarctic midge, especially those in the sugar and polyol pools, are adaptations that have potential to enhance survival during both cold and desiccation. Koštál *et al.* (2013) also found that the metabolic suppression of Snails in dry conditions probably included tissue hypoxia and anaerobism as indicated by accumulation of typical end products of anaerobic metabolism: lactate, alanine and succinate. But the technique has not been applied extensively to non-human primate studies.

Previous researches have paid more attention to the basal rate of metabolism and resting metabolic rates in non-human primate's metabolic researches (Schmid and Ganzhorn, 1996; Genoud *et al.*, 1997; Genoud, 2002; Kälin *et al.*, 2003; Power *et al.*, 2003). Several of the factors that have been introduced to explain the residual variation of the basal rate of metabolism among mammals are particularly relevant to primates, including accessibility to energy resources (McNab, 1986), climate (McNab, 2002) and nocturnality (Kurland and Pearson, 1986). These factors have already been discussed in several studies (Müller, 1985; Kurland and Pearson, 1986; Ross, 1992; Genoud, 2002). From an ecological point of view the low metabolic rates of prosimians are not an evolutionary primitive feature but rather represent a mechanism to cope with environmental constraints (Müller, 1985).

The Sichuan golden monkey (*Rhinopithecus roxellana*) is well-known as the non-human primate with the highest known altitudinal distribution. This species is on the list of class I protected animal in China. In recent decades, conservation efforts have received much attention with respect to evolution, distribution and ecology (Kirkpatrick, 1995; Kirkpatrick and Grueter, 2010; Sterck, 2012). Researches have shown that environmental changes and human activities affected the distribution and genomic divergence of the golden snub-nosed monkeys, knowledge of which will help improve conservation management strategies (Chang *et al.*, 2012; Luo *et al.*, 2012). Yet few reports have been fully initiated to monitor metabolic profiling in Sichuan golden monkeys. Our objectives were to identify all metabolites in Sichuan golden monkeys using GC-MS analysis of urine, and screen out (if possible) a number of important urinary metabolites related to gender differences and seasonal variations.

MATERIALS AND METHODS

Animals

Fifteen adult captive-bred Sichuan golden monkeys (ten males, M1–M10, five females, F1–F5) living in the Shanghai Wildlife Zoo in Shanghai, China (120° 52'–122° 12' E and 30° 40'–31° 53' N) were studied for this research. Ten of the monkeys (five males, M1–M5, five females, F1–F5) were housed together in an enclosure with an indoor night-time rest area measuring approximately 60 m^2, with access to an outdoor yard of about 900 m^2. The other five males (M6–M10) were housed individually in cages measuring approximately 12 m^2. The indoor enclosure had no heating nor air conditioning. And the temperature in the indoor enclosure was as the same as the ambient temperature. All animals were healthy during the experiment, and were fed with normal diet of fresh privet, mulberry and pittosporum leaves (about 40% of the total food weight) supplemented with eggs, steamed corn bread and vegetables. Water was available at all times. All experiments were carried out in accordance with the National Institutes of Health guidelines and Wildlife Protection Law of the PRC.

Sampling

Facial features, coat color and other physical characteristics of ten Sichuan golden monkeys recognized (F1–F5 and M1–M5) in order to confirm the target animals. Fresh urine of ten individuals was collected immediately after urination for one month (from Nov. 20 to Dec. 19, 2012). Fresh urine of five males (M6–M10) was collected on each of 30 days in every season (from Mar. 2012 to Mar. 2013). Four seasons were defined according to weather conditions and decade report: spring (from Mar. 24 to Jun. 4), summer (from Jun. 5 to Sep. 28), autumn (from Sep. 29 to Dec. 1), winter (from Dec. 2 to March 23). The ambient temperatures of four seasons were shown in Table I. All urine samples were collected between 0700–0900. All urine samples were labeled, placed in sterile centrifuge tubes, and stored at –70°C prior to processing.

Table I.- Ambient temperatures for each of the four seasons in study site.

The ambient temp. (°C)	Maximum	Minimum	Mean
Spring	21.14	14.98	17.80
Summer	30.18	25.11	27.24
Autumn	19.08	12.38	15.43
Winter	7.18	3.18	4.99

Preparation of urine samples

The vortexed urine pool was centrifuged at 4000 × g for 10 min to isolate the urine supernatant, sediment was removed. Urease (20 IU) was added to 100 µl of urine, and then were incubated at 37°C for 20 min to decompose any excess urea. Next, 300 µl of methanol (12.5 µg/ml, including the stable isotope-labeled internal standard compound [^{13}C2-]-myristic acid) was added into the solution. The mixtures were vortexed for 5 min and then allowed to stand for 1 h (4°C). They were centrifuged for 10 min (4°C, 20000 × g), and an aliquot (100 µl) of each mixture was transferred to a GC vial and dried in a Speed-Vac Concentrator. 30 µl of pyridine (methoxyamine grade, 10 mg/ml) was added to the GC vial, vortexed for 3 min. 30 µl of MSTFA (1% TMCS) was added, vortexed for 1 min, and allowed to conduct silylation at least at 75°C for at least 30 min. Finally, 30 µl of methyl myristate (30 µg/ml) was added to the final solution of each sample as the external standard to check for system variation.

GC-MS analysis

The derivatized sample (0.5 µl) was applied by splitless injection (1:10) using a Shimadzu GC-MS QP 2010 Ultra/SE (Kyoto, Japan) equipped with a fused silica capillary column (30 m × 0.25 mm i.d., J&W Scientific, USA). Helium was used as the carrier gas at a constant flow rate of 1.5 ml/min through the column, and the temperature-programmed mode was used: 80°C (3 min), 70–300°C; linear heating (20°C /min), 300°C (5 min). The injector temperature was set at 250°C; cleaning time and flow velocity to 1 min, 20 ml/min; transfer tube temperature to 220°C; ion source temperature to 200°C; and bombarding electron flow: –70 eV, detector threshold –950 V. Masses were scanned from 50 to 800 m/z at a rate of 2500 Hz, after a solvent delay of 300 s. In addition, pooled urine samples and blank sample (distilled water) were put through the same analysis as urine.

GC-MS data acquisition and identification of the compounds

The automatic peak detection and mass spectrum deconvolution using Agilent Ehanced ChemStation software were performed. To obtain accurate peak areas for the IS and specific compounds, two unique quantification masses for each component were specified, and the data were reprocessed. The retention index for each peak was calculated by comparing the retention times against those of a C8–C40 alkane series. The compounds were identified by comparison of the mass spectrum and retention index of each compound with reference standards and data available following in libraries: mainlib and publib, in the NIST (National Institute of Standards and Technology) Library (2008), and Wiley Chemical Structural Libraries (Excalibur software, Thermo Inc.) further confirmed or established metabolite identities.

Multivariate analysis

The resulting three-dimensional matrix was imported into the Simca-P 11 software package (Umetrics, Umeå, Sweden), including Principal components analysis (PCA) and Orthogonal partial least squares projection to latent structures and discriminant analysis (OPLS-DA). A data matrix was constructed with sample names/IDs as the observations and retention times of the response variables. Additionally, the concentration of each verified metabolite was quantified via the corresponding calibration curve and was expressed as the relative percentage of change. Statistical significance of the datasets was analyzed using SPSS version 16.0. The threshold of the *P* value was 0.05 throughout the study.

RESULTS

Metabolite identification

A total of sixty-one metabolites were detected in the urine samples of these Sichuan golden monkeys. These metabolites were present in detectable amounts, and their identities were verified using MS, including five amino acids, eight

sugars and polyols, fifteen fatty acids and hydroxy fatty acids, seven metabolic intermediates (Table II), one small molecule, and twenty-five metabolites that did not fall into the above categories (Table III).

Table II.- Various metabolites (amino acids, sugers and polyols, fatty acids and hydroxy fatty acids) in the urine samples obtained from Sichuan golden monkeys.

Classification of metabolites	Retention time (min)	Endogenous metabolites
Amino acids	5.55	Alanine
	5.72	Glycine
	7.83	Proline
	8.96	Proline
	9.53	Glutamic acid
Sugers and polyols	8.87	Erythritol
	10.13	Arabinitol
	10.34	D-ribose
	11.05	D-fructose
	11.19	Glucose
	11.37	Glucitol
	14.83	Maltose
	14.91	Lactose
Fatty acids and hydroxy fatty acids	5.13	2-hydroxybutyric acid
	5.25	Glycolic acid
	5.90	2-hydroxy-2-methyl butanoic acid
	6.13	3-hydroxyisobutyric acid
	6.49	2-methyl-3-hydroxy butyric acid
	6.53	4-hydroxybutyric acid
	6.59	Hydroxyisovaleric acid
	6.63	Methylmalonic acid
	6.76	2-ethyl-3-hydroxypropionic acid
	7.53	Pyrotartaric acid
	7.77	5-hydroxyhexanoic acid
	7.80	2,4-dihydroxy butanoic acid
	8.05	Mesaconic acid
	8.98	3-methyladipic acid
	9.11	Threonic acid
Metabolic intermediates	5.09	Lactic acid te
	6.79	Urea
	7.01	2-ketoglutarate
	7.43	Succinate
	8.71	Malic acid
	10.26	Cis-aconitic acid
	10.71	Citrate

Table III.- Various metabolites (small molecules and other metabolites) in the urine samples obtained from Sichuan golden monkeys.

Classification of metabolites	Retention time (min)	Endogenous metabolites
Small molecules	7.17	Phosphoric acid
Other metabolites	5.84	Oxalic acid
	6.91	Benzoic acid
	7.10	2-aminoethanol
	7.67	Uracil
	8.41	Pimelic acid
	8.76	Dihydrouracil
	9.21	Creatinine
	9.48	3-hydroxyphenylacetic acid
	9.58	4-hydroxybenzoic acid
	9.65	4-hydroxyphenyl acetic acid
	9.89	Taurine
	10.17	3-(3-hydroxyphenyl) propanoic acid
	10.43	Homovanillic acid
	10.76	3,4-dihydroxy phenylacetate
	10.80	Hippurate
	10.98	Quinic acid
	11.11	Allantoin
	11.13	Hydroxyphenyllactic acid
	11.41	Glucuronic acid
	11.50	Glucaric acid
	11.57	D-gluconic acid
	11.61	Vanylglycol
	12.09	N-acetyl glucosamine
	12.14	Uric acid
	12.30	3-hydroxyhippuric acid

Metabolic response to gender differences

Two groups (five males vs. five females) were not completely separated from each other in the PCA scores plot of urine (Fig. 1A). However, these two groups were distributed in two areas of the OPLS-DA scores plot (Fig. 1B). The changes in the concentration of various metabolites for the urine samples obtained from the females and males are presented in Table IV, in the form of the mean ± standard error. The levels of alanine, glutamic acid and taurine in the females were higher than in the males ($P<0.05$); however, the levels of methylmalonic acid, 5-hydroxyhexanoic acid and 2-ketoglutarate in the males were higher than in the females ($P<0.05$).

Metabolic response to seasonal variations

The score plot of the urine samples illustrated the metabolic response of Sichuan golden monkeys to seasonal variations. Four groups were not completely separated in the PCA scores plot of urine (Fig. 1C). Then, the OPLS-DA scores plot showed that there were separation trends among the four seasons, and no overlap in the analytical outcomes was observed among the four seasons (Fig. 1D). The changes in the concentrations of various metabolites for the urine samples obtained from the four seasons are presented in Table V, in the form of mean ± standard error. The levels of the tricarboxylic acid (TCA) cycle intermediates (citrate, 2-ketoglutarate, malic acid and succinate) and creatinine in the urine during the summer was significantly lower than in the other three seasons ($P< 0.05$), and the levels of these metabolites in the winter were significantly lower than in the autumn and spring ($P< 0.05$). The urinary alanine levels in the summer and winter were significantly lower than in the autumn and spring ($P< 0.05$). Additionally, the levels of glycine, serine, hippurate and benzoic acid in the urine were the lowest in the summer ($P< 0.05$), and there were no differences in these metabolite levels between the other three seasons.

Table IV.- Differences in metabolites of urine from female and male Sichuan golden monkeys (mean±SEM).

Metabolites	Female	Male
Alanine	0.31±0.01[a]	0.19±0.03[b]
Glutamic acid	0.22±0.01[a]	0.14±0.01[b]
Taurine	0.19±0.01[a]	0.08±0.00[b]
Methylmalonic acid	0.48±0.03[a]	0.62±0.02[b]
5-hydroxyhexanoic acid	0.02±0.00[a]	0.03±0.00[b]
2-ketoglutarate	0.06±0.00[a]	0.07±0.00[b]

[a,b]: P < 0.05, significant difference in metabolites of urine from female (n = 5) and male (n = 5) Sichuan golden monkeys. Values with different letters in the same line showed significant difference.

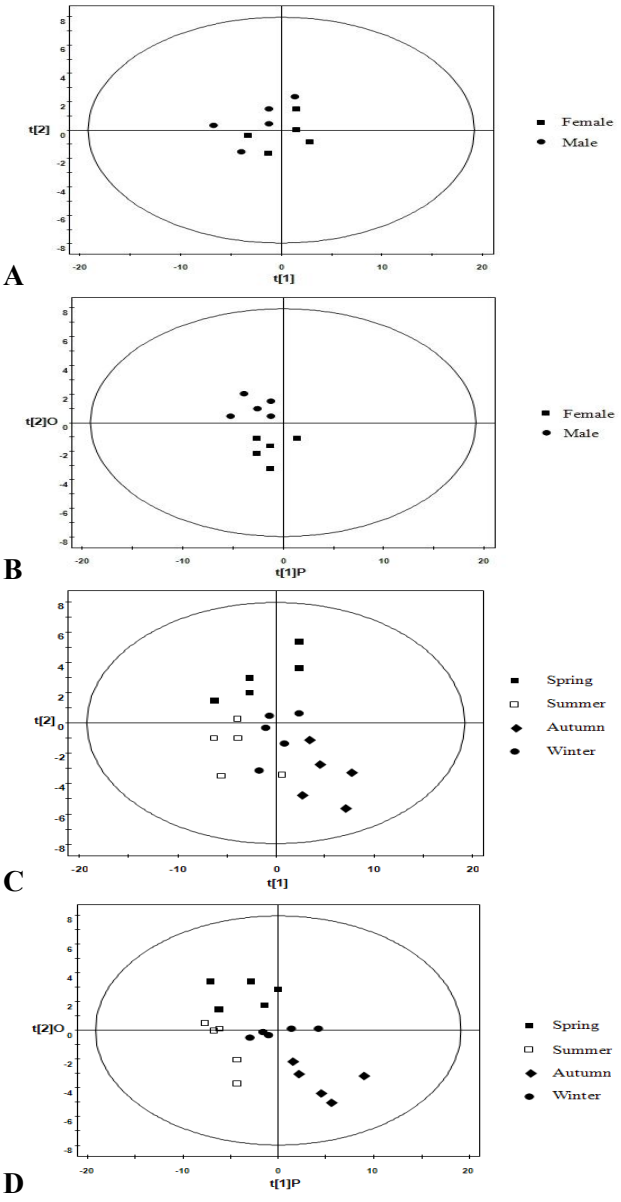

Fig. 1. The PCA scores plot (A) and OPLS-DA scores (B) plot of urinary data comparing female and male Sichuan golden monkeys (n = 10). PCA scores plot (C) and OPLS-DA scores plot (D) of urinary data obtained from seasonal variation-induced male Sichuan golden monkeys (n = 5).

DISCUSSION

Twenty-four and thirty-eight metabolites, were identified in normal human urine and police dog plasma respectively by this approach (Adamko *et al.*, 2007; Liu, 2009). Yet, no study has been done on the metabonomics for the urinary metabolites of Sichuan golden monkeys. Sixty-one endogenous metabolites were identified in the urine samples of these Sichuan golden monkeys. Therefore, we obtain a comprehensive view in

Table V.- Changes in the urine metabolites of Sichuan golden monkeys, induced by seasonal variations (Mean±SEM).

Metabolites	Spring	Summer	Autumn	Winter
Citrate	1.64±0.03^{c}	0.63±0.03^{a}	1.76±0.06^{c}	1.15±0.09^{b}
2-ketoglutarate	0.12±0.01^{c}	0.05±0.01^{a}	0.13±0.01^{c}	0.08±0.01^{b}
Malic acid	0.14±0.01^{c}	0.02±0.00^{a}	0.14±0.01^{c}	0.10±0.01^{b}
Succinate	4.04±0.59^{c}	0.87±0.04^{a}	3.11±0.18^{c}	1.89±0.12^{b}
Creatinine	1.10±0.03^{c}	0.41±0.05^{a}	1.21±0.03^{c}	0.86±0.13^{b}
Alanine	1.39±0.13^{b}	0.78±0.02^{a}	1.67±0.15^{b}	0.50±0.03^{a}
Glycine	0.22±0.03^{b}	0.02±0.00^{a}	0.17±0.08^{b}	0.24±0.06^{b}
Serine	0.11±0.00^{b}	0.05±0.00^{a}	0.14±0.03^{b}	0.16±0.02^{b}
Hippurate	2.52±0.15^{b}	1.48±0.05^{a}	2.67±0.12 ^{b}	3.03±0.64^{b}
Benzoic acid	7.46±0.59^{b}	5.04±0.11^{a}	7.28±0.17^{b}	8.52±0.77^{b}

^{a,b,c}: $P < 0.05$, significant difference in the metabolites of the urine samples obtained from seasonal variation-induced male (n = 5) Sichuan golden monkeys. And values with different letter in the same line showed significant difference.

abundance of numerous compounds simultaneously, which may provide a theoretical basis for physiological status and biochemical adaptations to environmental stressors in this species.

Metabolomic variations caused by gender differences

Recently, human urine studies indicated that gender has impact on metabolite excretion (Adamko *et al.*, 2007). The previous study on *Lepilemur ruficaudatus* suggested that there is no difference in mean resting metabolic rates between the sexes (Schmid and Ganzhorn, 1996). However, the researchers do not analyze the specific metabolic mechanism related to gender in non-human primates. In this study, there was significant difference in metabolite excretion between male and female Sichuan golden monkeys. The utilization ratio of amino acid (alanine and glutamic acid) in females may be lower than in males, because the amino acid contents in the urine have an inverse ratio with protein synthesis. Taurine is involved in bile acid biosynthesis. So the difference of taurine level between females and males indicates that there may be difference in lipid metabolism between two genders. Methylmalonic acid is a vital intermediate in the metabolism of fat and protein, and 5-hydroxyhexanoic acid is a normal dicarboxylic acid degradation product of fatty acids. Besides, 2-ketoglutarate is a key intermediate in the Krebs cycle; therefore, the differences in the above three metabolites indicate that there are differences in energy metabolism between males and females. A number of metabolic changes of this study further imply that an intrinsic physiological difference between males and females in this species.

Metabolomic variations caused by seasonal variations

In some cases, fluctuating temperatures on both a daily and seasonal scale may become severely stressful or even lethal; therefore, animals develop a number of physiological, biochemical and behavioral adaptations that allow them to overcome such stressful situations (Malmendal *et al.*, 2006; Seki, 2013). To obtain a better understanding of the effects of stressful situations on metabolite concentrations in the organism, it is relevant to adopt an integrative approach, such as a simultaneous measurement of the overall effects of stress or biological perturbation, *e.g.,* using the GC-MS technique. The present study investigated the overall and specific changes in the Sichuan golden monkey's metabolome following seasonal variations. And the variability results for the metabolites with seasonal variations are worthy of particular discussion.

Several metabolic intermediates (citrate, 2-ketoglutarate, malic acid and succinate) in the urine were altered by seasonal variations. It has been well documented that citrate, 2-ketoglutarate, malic acid and succinate are the crucial substances of TCA cycle, which is the main pathway of glucose degradation and is primary energy supplier for universal organisms (Ni *et al.*, 2007). As reported earlier, irrespective of any kind of physiological

stress, there is increased energy consumption and protection against internal and external stress provided by allostasis (ability to achieve stability through change) (Kulinskiĭ *et al.*, 1986; Robert *et al.*, 2008). Pereira (1993) suggested seasonal suppression of metabolic rate in large diurnal lemurs, and took this as an analogous tactic to torpor in non-primate mammals. Other species also show the corresponding physiological response to the environmental stress, for example, the urinary excretion levels of several TCA cycle intermediates decrease following acute heat stress for rats (Gandhi *et al.*, 2011), the accumulation of succinate is related to the metabolic suppression of Snails in dry conditions (Koštál *et al.*, 2013), sorbitol providing resistance to high temperatures in whiteflies (Wolfe *et al.*, 1998) or glucose protecting against freezing damage in frogs (Costanzo *et al.*, 1991). In this study, reduced metabolites involved in energy metabolism may be caused by a general increase in the metabolic rate because of the elevated temperature in the summer, and the TCA cycle is accelerated due to enhanced adrenergic nerve activity. Then in the winter, the levels of TCA cycle intermediates in the urine were significantly lower than in the spring and autumn, suggesting that cold stimulation causes increased muscle tension, ultimately accelerating energy metabolism. In the spring and autumn, Sichuan golden monkeys appeared to have lower energy consumption periods, and the TCA cycle intermediates may reflect a stabilized state of whole-body oxidative energy metabolism. Hence, the alteration of the TCA cycle in Sichuan golden monkeys is an important part of metabolic regulatory and compensatory mechanisms in response to external environmental stress. In addition, the basic information on energy metabolism in Sichuan golden monkeys may indicate their physiological adaptations and survival to seasonal flucuations of abiotic conditions.

In this study, the creatinine levels of the urine were relatively low in the summer and winter. Reduced creatinine may be indicative of reduced glomerular filtration rate and/or modifications of transport mechanism at tubular level which may be related to altered cellular function or low glucose in tubular lumen. Reduced creatinine levels might also indicate reduced ability of kidney to eliminate acids and may be considered as an early marker for impaired renal function (Gandhi *et al.*, 2011).

The free amino acid pool (alanine, glycine and serine) in the urine was also considerably altered by seasonal variations. Alanine is related to energy metabolism. Additionally, alanine can protect proteins from cold inactivation (Carpenter and Crowe, 1988). Alanine upregulation *in vivo* has been correlated with a number of insect cold-hardy states (*e.g.*, Kukal *et al.*, 1991; Fields *et al.*, 1998; Rivers *et al.*, 2000; Goto *et al.*, 2001). In this study, the reduction of urinary alanine in the winter and summer may be the result of rapid utilization of energy caused by a temperature-dependent increase in metabolism. Two other free amino acids, glycine and serine levels in the urine were the lowest in the summer. Glycine and serine are linked in the same biosynthetic pathway, thus a decrease of glycine coupled with a marked reduction in serine indicates that the activity of one or both of these pathways is affected by heat stress in the summer or one of the pathways using these two amino acids as a substrate is activated. Therefore, a number of amino acid changes in Sichuan golden monkeys may be adaptations that have potential to enhance survival during both heat and cold.

Mammalian metabolism is known to be significantly influenced by complex gut microbial community interactions (Xu *et al.*, 2003). Changes in hippurate and aromatic metabolites are closely correlated to the activities of gut microflora (Phipps *et al.*, 1998; Gonthier *et al.*, 2003; Nicholls *et al.*, 2003; Williams *et al.*, 2005; Dumas *et al.*, 2006; Gao *et al.*, 2011). In this study, the levels of hippurate and benzoic acid were decreased in the summer. Summer temperatures attain yearly peaks and microbial load and variety (principally in food) are highest in this season, indicating significant involvement of the gut microbiota in response to heat stress, and decreased excretion of urinary hippurate and benzoic acid may also be associated with a disturbance of microbial colonies (Nicholls *et al.*, 2003). The urinary excretion level of hippurate is decreased following acute heat stress for rats (Gandhi *et al.*, 2011). These observed effects on gut microbiota are interlinked with stress-induced variations of catecholamines and noradrenaline, as they coexist with the gut microflora in the

gastrointestinal tract (Lyte and Bailey, 1997; Hawrelak and Myers, 2004). In the other three seasons, no difference was observed in the hippurate and benzoic acid levels, indicating that the gut microbial community interactions of Sichuan golden monkeys were relatively balanced. Thus, changes in hippurate and benzoic acid caused by seasonal variations may result from the microbial load, thereby causing a disruption in microbial populations and hence metabolism in this species.

The current GC-MS platform successfully identified endogenous components in Sichuan golden monkey urine. Therefore, we obtained a preliminary understanding of the metabolic mechanisms of Sichuan golden monkeys. And a number of metabolic changes indicate underlying adaptation and survival of these monkeys to extraneous environmental factors in their natural environment and habitats, which may aid appropriate conservation management strategies in this speices. Future studies are required on direct biomarkers for external insults in this species and other wildlife.

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