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Supplementation with turmeric residue increased survival of the Chinese soft-shelled turtle (*Pelodiscus sinensis*) under high ambient temperatures*

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Abstract: Turmeric residue (TR), containing residual levels of curcumin, is a solid by-product waste generated after the extraction and separation of curcumin from turmeric root. A feeding trial was conducted to evaluate the effects of TR on the survival of Chinese soft-shelled turtles (SSTs), *Pelodiscus sinensis*, under a high ambient temperature. A total of 320 female SSTs were assigned randomly to two diets: basal diet (the control group, n=160) and an interventional diet supplemented with 10% TR (the TR group, n=160). Our results demonstrated that supplementation of TR increased the SST survival rate by 135.5%, and superoxide dismutase (SOD) activity of SST liver by 112.8%, and decreased the malondialdehyde (MDA) content of SST liver by 36.4%, compared to the control group. The skin of the SST fed TR showed a golden color. High-performance liquid chromatography (HPLC) analysis indicated that the concentrations of curcumin in TR and the skin of the SST fed TR were (1.69±0.30) and (0.14±0.03) μg/g, respectively. Our observation suggests that supplementation of TR increased the survival rate of SST under high ambient temperatures. We speculated that the increased survival rate and tolerance at the high ambient temperature were associated with the anti-oxidation activity of curcumin from TR. Moreover, curcumin in TR could be deposited in SST skin, which made it more favored in the market of China. Our findings provide new knowledge and evidence to effectively reuse TR as a feed additive in animal and aquatic farming.

Key words: Turmeric residue (TR); Curcumin; Chinese soft-shelled turtle (SST); Survival; High ambient temperature; Antioxidant
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1 Introduction

The Chinese soft-shelled turtle (SST), *Pelodiscus sinensis* (Wiegmann), is an important member of the reptilian aquaculture with high nutritional and medicinal value (Chen et al., 2014). SST has been widely used as a nutraceutical for treating diabetes, the menopause, hypertension, and anemia, and other

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chronic diseases in China (Chen CY and Huang, 2015). On the China mainland, 285 900 tons of SSTs were produced and consumed in 2012 (Chen CY and Huang, 2015). However, the SST is suffering from unhealthy farming practices, such as the use of chemical drugs, high-density culturing, and environmental pollution, resulting in significant reduction in both quality and quantity, high mortality rate, and serious economic losses in aquatic farming.

To increase the survival rate and improve the quality of the SST, the effects of nutrients, such as lipids (Perez-Casanova et al., 2010), vitamin A (Chen LP and Huang, 2015), vitamin C (Wang and Huang, 2015), vitamin E (Zhou et al., 2004), magnesium (Chen LP and Huang, 2015), and probiotics (Zhang et al., 2014), on feeding SST have been investigated in recent years. At the same time, the effects of farming environmental factors such as incubation temperatures (Dang et al., 2015), *Bacillus thuringiensis* fatal infections (Chen et al., 2014), and acute ammonia toxicity (Ip et al., 2008) on SST were also observed. However, the effects of natural herbal supplementation, like curcumin, on the survival rate of the SST under a high ambient temperature and resistance to infection have received little attention.

Turmeric (*Curcuma longa* L.), a yellow-colored rhizomatous herbaceous perennial plant of the Zingiberaceae family, grows natively in the tropical regions of southern and southeastern Asia. Turmeric root containing turmerin, essential oils, and curcuminoids including curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) has been used in traditional medicine in Asia for more than 2000 years (Shen et al., 2013b). Curcumin, extracted from turmeric root, is a main bioactive polyphenol that has been used widely as a spice, food additive, and as an herbal medicine in Asia. Numerous studies have demonstrated that curcumin possesses diverse biological functions that combat inflammation, diabetes, spasticity, allergy, rheumatism, and cancer (Sharma et al., 2009; Shen et al., 2013a; Zhang et al., 2013). In recent years, curcumin as a potential therapeutic agent in treating diabetes-associated disorders, like neuropathy, nephropathy, and vascular disease has attracted much scientific attention (Zhang et al., 2013). In addition, work has been carried out to understand the molecular mechanisms of the antioxidant properties with experiments in vivo and in vitro (Shen et al., 2013b).

Turmeric residue (TR) containing residual levels of curcumin, is a solid by-product waste generated after the extraction and separation of curcumin from turmeric root. With the rapid development of the turmeric industry, a tremendous amount of TR waste is produced every year. There is a strong desire to find a way to make use of this industrial waste. Curcumin as a feed additive has been used in the animal food industry because of its beneficial effect on domestic animals such as broiler chicks (Yarru et al., 2009; Zhou and Luo, 2013). However, the high cost of using curcumin directly as supplementation prevents it being of practical use in animal farming. In addition, the usage, which has been tentative, of TR to improve the health of domestic animals including SST has not been sufficiently documented.

According to a report from the American Meteorological Society (Blunden and Arndt, 2014), 2013 was the warmest year since 2010 and one of the ten warmest years since records began in the mid- to late 1800s. The mean annual temperature over China was 10.2 °C, 0.6 °C above normal, the fourth highest since 1961, and 0.8 °C higher than that of 2012. The seasonal mean surface temperatures (anomalies) were 21.7 °C (+0.8 °C) and 10.5 °C (+0.6 °C) for summer and fall, respectively (Blunden and Arndt, 2014). By coincidence, a dietary intervention with TR in SST was conducted in the summer of 2013 in Haiyan, China, and this provided a rare opportunity to investigate the effects of TR on the SST survival rate at a high temperature and elucidate the possible mechanisms of TR in SST.

2 Materials and methods

2.1 Reagents

The curcumin standard was purchased from Shanghai Winherb Medical Science Co., Ltd. (Shanghai, China). Kits of malondialdehyde (MDA) (Cat. A003-1) and superoxide dismutase (SOD) activity (Cat. A001-2), and protein were purchased from Nanjing Jiancheng Bioengineering Company (Nanjing, China). Methanol and acetonitrile were purchased from Tjshield (Tianjin, China). Acetic acid and ethyl acetate were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All chemicals used were either of high-performance liquid chromatography (HPLC) grade or of analytical grade.

2.2 Animals

The experiments were conducted in accordance with the standards at Yucheng Turtle Farm of Haiyan, China from June 19 to Oct. 20 in 2013 (4 months). Juvenile female SSTs of 24-month age with an approximate body weight of 250 g were raised from the same hatching day by the farm. A total of 320 healthy young female turtles were assigned randomly into two groups, the control group and TR group (the intervention). Each group of 160 turtles was raised in an outdoor pool of 32 square meters. These pools are enclosed with cement walls of 1.5 m in height to prevent turtles from escaping.

2.3 Culture conditions

The natural culture environmental conditions were the same in both the control and interventional pools, which were set 5.8 m apart. Fresh water was added to each pool every morning. The temperature records including average, maximum, and minimum from July to Sept. of 2013 were provided by Haiyan Weather Station, China.

2.4 Diets

TR provided by Ningbo Traditional Chinese Pharmaceutical Co., Ltd. (Ningbo, China) was milled into 60 mesh powder. A total of 800 g turtle feed per pool was prepared daily into granules in accordance with a standard diet formula which contained fish 62%, wheat starch 22%, soybean meal 7%, yeast powder 5%, vitamin mix 2%, and mineral 2% (w/w) (Zhang et al., 2014), and spread into each pool allowing turtles to ingest freely twice (400 g in the morning and 400 g in the evening) every day. The basal diet (control) contains 65% of marine fish powder, 24% of starch, and 2% of yeast, salt, amino acids, vitamins and minerals. The interventional diet contained 90% basal diet and 10% TR powder in weight.

2.5 Growth and survival rate

Each turtle was weighed before the trial on June 19, 2013. The final surviving turtles were counted and weighted on Dec. 11, 2013 when all live turtles of both control and TR groups were collected after draining all the water from the pools. It had not been possible to determine the exact number of live and dead turtles during the experiment. The survival rate was calculated according to the formula: survival rate (%)=(final count of survival turtles/initial count of turtles)×100%.

2.6 HPLC analysis of curcumin in TR and turtle skin

Ethyl acetate (95%, v/v) and methanol (5%, v/v) were used to extract curcumin from TR and turtle skin. The solvent and sample were mixed at the ratio of 10:1 (v/w), and then samples were treated by ultrasonic for 30 min. Then the mixtures were centrifuged at 5000 r/min for 10 min and filtered through a 0.45-μm filter membrane. The filtrate was concentrated with nitrogen to remove the organic solvent. The dried samples were re-dissolved in 5 ml of mobile phase and vortex-mixed for 30 s; then, 1.5 ml of the sample was transferred into an injection sample vial.

Curcumin standards at the concentrations of 0.01, 0.05, 0.10, 0.50, 1.00, and 2.00 μg/ml were prepared and the calibration curve was used to determine the concentrations of curcumin in samples. Each sample was prepared at least in three independent replicates.

The analytical method determining curcumin concentrations in TR and turtle skin was described by Schiborr et al. (2010) with slight modification. The chromatographic analysis was carried out by an Agilent HPLC system using Eclipse XDB-C18 column (4.6 mm×250 mm, 5 μm) (Agilent Technologies, Santa Clara, California, USA) with a mobile phase composed of 49% acetonitrile, 20% methanol, 30% deionized water, and 1% acetic acid (v/v). Flow rate was set to 0.4 ml/min. The column temperature was maintained at 25 °C. A volume of 10 μl was injected into the HPLC system. The absorbance of the detector was set at 430 nm (Shen et al., 2013b).

2.7 Anti-oxidation assay

Liver samples were collected from turtles of both control and TR groups. Twelve samples from each group were frozen in liquid nitrogen and then stored at −80 °C for future analysis. MDA levels and total SOD activity were measured separately using an MDA kit and a SOD kit according to the instructions provided by the manufacturers. The level of lipid peroxidation was expressed as mmol MDA per mg protein. One unit of SOD activity was defined as the amount of enzyme required to cause a 50% inhibition and expressed as enzyme units per gram of the total protein.

2.8 Statistical analysis

Statistical analysis was conducted using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Excel (Microsoft, Redmond, WA, USA) was used to express graphical presentations. In the anti-oxidation assay, the levels of MDA and SOD were estimated at least in three independent replicates, and results were reported as mean±standard deviation (SD). The difference in survival rate between the control and TR group was examined using Student's *t*-test, with *P*-value less than 0.05 being statistically significant.

3 Results

3.1 Effects of feeding TR on survival rate and weight of turtles under high ambient temperature

Table 1 shows the final survival rate and mean weight of turtles from both the control and experimental groups. The final survival rates of the TR and control groups were 86.9% and 36.9% ($P<0.0001$), respectively; the survival rate of the former was increased by 135.5% compared to that of the latter. However, there is no statistically significant difference in the final mean weight between the control and TR groups: (504.9±125.8) g and (481.9±141.3) g ($P=0.096$), respectively. On the other hand, the total weight of the TR group is more than double of that of the control (67 kg vs. 30 kg).

As high temperatures could cause increase in the death rate of turtles (Li et al., 1995), we analyzed the local climate data from July to Sept., 2013, and found that the summer of 2013 in Haiyan had a successive 48 d (from July 1 to Aug. 17, 2013) of high temperatures with the maximum temperature >34 °C. In particular, a daily maximum temperature above 40 °C persisted for 5 d during Aug. (Fig. 1). This could have resulted in a higher death rate of SST in the control group. However, we could not determine the pathological cause of turtle death in the present experiment.

3.2 Curcumin content and the skin color of turtles fed TR diet

The turtle skin of the control group is incarnadine (Fig. 2a), whereas the color of the turtle skins from the TR group looks golden, a color resembling that of curcumin (Fig. 2b). To determine if the golden color of the TR is correlated with curcumin of TR, we conducted HPLC analysis of the turtle skin from both groups.

Table 1 Effect of TR on the survival of the soft-shell turtle

Group	Initial count (turtle)	Final survival count (turtle)	Mean weight[1] (g/turtule)	Total weight[2] (kg/pool or group)	Survival rate[3] (%)
Control	160	59	504.9±125.8	29.79	36.9
TR	160	139	481.9±141.3	66.99	86.9
P-value			0.096		<0.0001

[1] Mean weight of each turtle was calculated as the average weight of all survival turtles per pool; values are expressed as mean±SD. [2] Total weight was the total individual weight of all survival turtles per pool. [3] Survival rate (%)=(final count of survival turtles/initial count of turtles)×100%

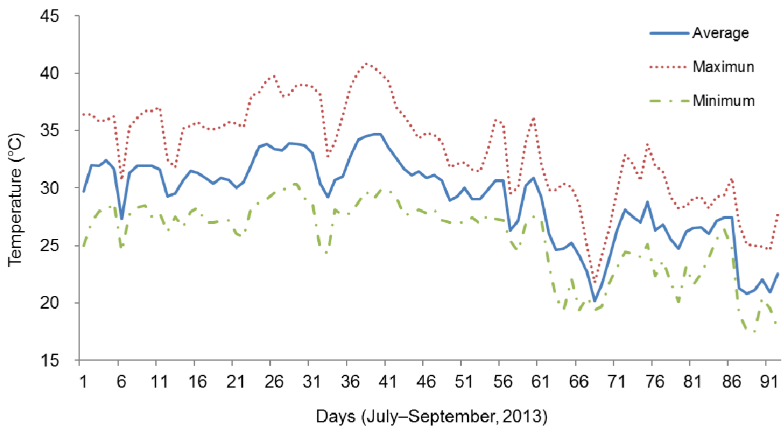

Fig. 1 Daily ranges of temperatures in minimal, maximal, and mean temperatures experienced by the *P. sinensis* from July to Sept., 2013

The HPLC retention time of curcumin was measured approximately at 6.18 min (Fig. 3a). The standard curve for curcumin was highly linear (R^2>0.99) within the range of 0.01–2.00 μg/ml. Our results demonstrated that the concentration of curcumin in the TR was (1.69±0.30) μg/g (Fig. 3b). The skin of turtle fed TR diet also contained curcumin (0.14±0.03) μg/g (Fig. 3c), but no curcumin was detected in the skin of turtles fed the basal diet (Fig. 3d). All character chemical retention times of the skin of the turtle fed the TR diet and that of TR were identical to the standard (Fig. 3). These observations indicated that the golden color of the turtle skin in the TR group had come from the curcumin in TR.

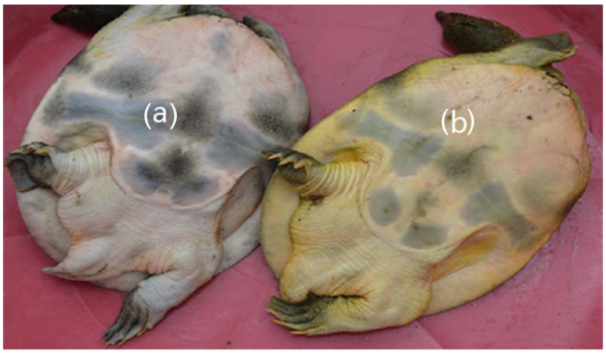

Fig. 2 Comparison of the turtle color from the control and TR groups
(a) A turtle from the control group; (b) A turtle from the TR group with the golden color

3.3 Effects of TR supplementation on SOD activity and MDA content in turtle livers

The SOD activity of the turtle livers from the TR group and control group was (2.76±0.14) and (1.30±0.32) U/mg protein, respectively, with the former being higher than the latter by 112.3% (*P*=0.0019; Fig. 4). The MDA content of the turtle livers from the TR and control groups was (0.07±0.01) and (0.11±0.01) nmol/mg pretein, respectively. The former was significantly less than the latter by 57.1% (*P*=0.0062; Fig. 5).

4 Discussion

Temperature is probably the most important factor for the survival of ectothermic vertebrates, especially for SSTs (Dang et al., 2015). Previous studies showed that incubation temperature may affect the immune function (Dang et al., 2015), hatching success, size, and morphology (Du and Ji, 2003) of SSTs. High temperatures decrease the resistance of reptiles to disease under global warming scenarios (Dang et al., 2015). The turtle's hatchlings from 26.5 °C grew significantly faster than did those from 34 °C (Du and Ji, 2003). The optimal ambient temperature for SSTs development ranges from 25 to 30 °C. Water temperature exceeding 40 °C or below 0 °C will cause death of SSTs (Li et al., 1995). In this study, we

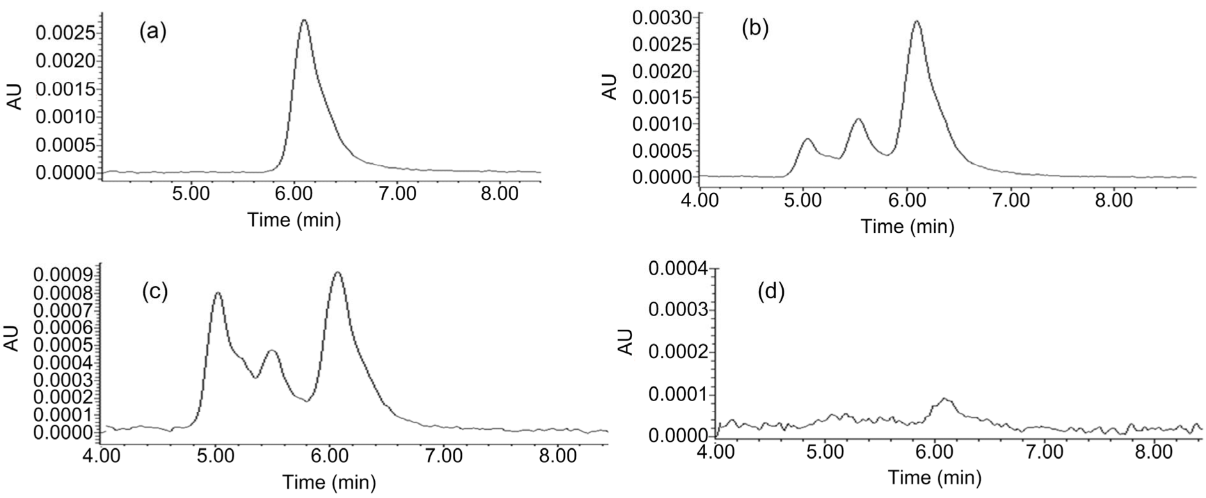

Fig. 3 HPLC chromatograms of curcumin standard, TR, and the turtle skins from the control and TR groups
(a) HPLC chromatograms of the curcumin standard; (b) HPLC chromatograms of curcumin from TR; (c) HPLC chromatograms of curcumin from the turtle skin of the TR group; (d) HPLC chromatograms of curcumin from the turtle skin of the control group

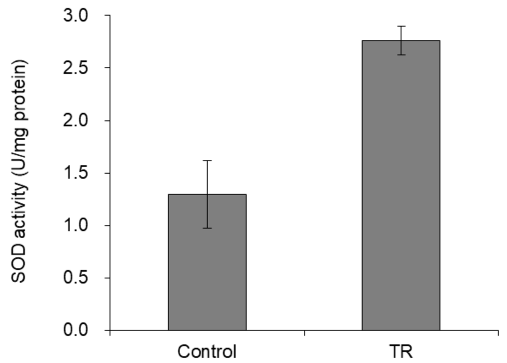

Fig. 4 Effect of TR diet on SOD activity of the turtle liver
Data are expressed as mean±SD with n=5, P=0.0019

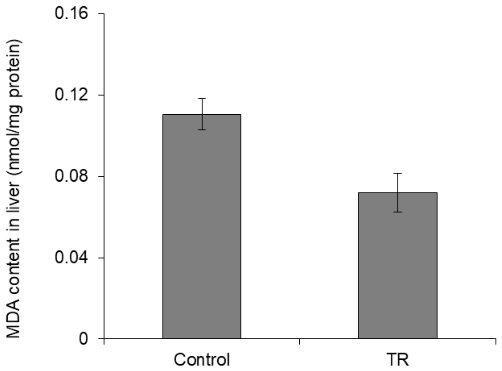

Fig. 5 Effect of TR diet on MDA content of the turtle liver
Data are expressed as mean±SD with n=5, P=0.0062

showed that the survival rates of soft-shelled turtles fed TR were more than double those of the control animals under a high ambient temperature. Our findings imply that supplementation of TR increased the ability of SST to resist the hazard of high temperatures.

On record at the global scale, 2013 was one of the 10 warmest years both at the Earth's surface and through the troposphere (Blunden and Arndt, 2014). A persistent heat wave occurred from the beginning of July to the end of Aug. across most of southern China. More than 300 stations exceeded a daily maximum temperature of 40 °C during the period (Blunden and Arndt, 2014). The similar seasonal mean surface temperature and persistent heat wave also occurred at Haiyan, China, from the beginning of July to the end of Aug. in 2013. Moreover, a daily maximum temperature exceeding 40 °C persisted for five days in Aug. in this year. Therefore, the high ambient temperature in the warmest year 2013 could result in high death rates of SST in the control group, providing us with a fortunate chance to conduct a dietary intervention with TR in SST. In addition, it should be indicated that we could not conduct a survivorship analysis with the feeding trial because of the difficulties in recording the exact time of death for each dead SST under water.

Curcumin possesses a strong intrinsic antioxidant activity against the oxidative stress environment (Shen et al., 2013a; Mahmood et al., 2015). It can directly affect a few major molecular targets, such as quenching reactive oxygen species (ROS) generated by mitochondria and elimination of ROS production (Shen et al., 2013a). Curcumin can suppress the pro-inflammatory state through inhibition of nuclear factor-κB (NF-κB)/mammalian target of rapamycin (mTOR) signaling pathways (Shen et al., 2013b), and inhibit the activity of the drug-metabolizing enzymes cytochrome P450, glutathione *S*-transferase, and uridine 5'-diphospho-glucuronosyltransferase (Sahin et al., 2012). Hence, supplemental dietary curcumin could protect the liver against injury and fibro-genesis by suppressing hepatic inflammation, attenuating hepatic oxidative stress (Mathuria and Verma, 2007), increasing expression of the xenobiotic detoxifying enzymes (Garcia-Nino et al., 2013), inhibiting activation of hepatic stellate cells, and supporting the mitochondrial function (Subudhi et al., 2008). In recent years, curcumin has become the subject of intense investigation in vitro and in animal models because of its potential health benefit (Chin et al., 2014). We have found that supplemental curcumin could increase mean lifespan via increasing SOD activity and decreasing MDA levels in *Drosophila* (Shen et al., 2013b).

In the present study, we observed an increased SOD activity in the livers of SSTs of the TR group when compared to that of the control, supporting the antioxidant properties of curcumin supplementation. On the other hand, the heat stress generates ROS, possibly by disrupting the electron transport assemblies of the membrane. The oxidative stress and the heat stress adversely affect the structure and physiology of the cell, causing impairment of transcription, RNA processing, translation, oxidative metabolism, and membrane structure and function. Heat shock response is another strategy for organisms to detect and respond to environmental, pathological or physiological stress and to control cell death and survival

(Teiten et al., 2009). The response can be induced by nonthermal stimuli such as heavy metals, sodium arsenite, prostaglandins, and oxidants (Dunsmore et al., 2001). Curcumin is capable of inducing the gene expression of heat shock protein (HSP), which is associated with its anti-inflammation activity (Teiten et al., 2009). It alleviates oxidative stress through modulating the hepatic nuclear transcription factors and HSP 70 in heat-stressed quails. Thus, supplementation of TR in SST diet could have activated the heat-shock response, which leads to the reduction of oxidative stress and increased survival rates of SSTs. However, the molecular mechanism, such as the effects on the expression of HSPs for curcumin in SSTs should be further investigated.

5 Conclusions

The present study demonstrated that SST feed supplementation with TR significantly increased the survival rates of SSTs under high ambient temperatures. HPLC analysis of TR and turtle skin showed that the active compound is curcumin from TR. The beneficial effects of curcumin on turtles are likely due to the increase of SOD activity and decrease of MDA. Our findings that supplemental TR in SST feed increased survival rates via increasing SOD activity and decreasing the accumulation of MDA, will provide new knowledge to use TR as a feed additive in the animal industry and aquaculture. Moreover, the deposit curcumin in the turtle skin showing golden color, a preferred color by consumers, provides a new route to increase the economic value of SSTs. It should be noted that these findings could also have application to humans. Turtles in general have high life expectancy for their size (about 50 years in the case of SSTs). Therefore, this animal could be used as a model of healthy aging in humans and it will be interesting to see if these findings apply to humans. Moreover, it remains to be seen whether consumption of turtles supplemented with TR could have additional benefit effects in humans compared with the consumption of turtles fed with a standard diet.

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Compliance with ethics guidelines

Yong CHEN, Yi-fan ZHANG, Hao-cheng QIAN, Jing-liang WANG, Zhe CHEN, Jose M. ORDOVAS, Chao-qiang LAI, and Li-rong SHEN declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：饲料中添加姜黄渣对高温环境下养殖中华鳖存活率的改善作用

目　的：评估饲料中添加姜黄渣对中华鳖存活率及抗高温应激能力的改善作用；通过中华鳖的抗氧化能力分析，探讨姜黄渣的生化作用机制。

创新点：证实了饲料中添加姜黄渣对中华鳖具有增强抗高温能力，提高存活率的作用，为姜黄渣作为新的水产饲料添加剂的资源开发应用提供了重要科学依据。

方　法：将 320 只雌性中华鳖（体重约 250 g）随机分为对照组（标准饲料）和实验组（饲料中添加 10% 的姜黄渣）。在 2013 年 7 月到 10 月分别考察实验组和对照组中华鳖的体重、存活率；分析试验地海盐县的气温变化与中华鳖存活率的相关性；用超氧化物歧化酶（SOD）和丙二醛（MDA）试剂盒分别检测了两组中华鳖肝组织的 SOD 酶活力及 MDA 含量；用高效液相色谱分别测定比较了两组中华鳖皮肤的姜黄素含量。

结　论：补充姜黄渣的中华鳖皮肤呈现金黄色，皮肤所含色素为姜黄素，平均含量为 (0.14±0.03) μg/g。存活率调查显示，实验组的中华鳖存活率比对照组高 135.5%；SOD 和 MDA 分析显示，实验组中华鳖肝组织的 SOD 酶活比对照组高 112.8%，MDA 含量比对照组低 36.4%。根据气温资料分析推测，持续高温容易导致中华鳖死亡，补充姜黄渣可有效降低高温造成的死亡率。综合分析显示，补充姜黄渣能显著提高中华鳖的存活率及抗高温应激能力，其作用与姜黄渣所含姜黄素的抗氧化活性相关。

关键词：姜黄渣；姜黄素；中华鳖；存活率；高温环境；抗氧化