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The Effects of Water Depth and Substrate Conditions on Changes in Body Weight and Health Status of Captive Big-headed Turtles (*Platysternon megacephalum*) during Hibernation

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Authors' Contribution
SG conceived and designed the experiments. JT, YC and JD performed the experiments. LH analyzed the data and wrote the manuscript. XZ contributed the experimental materials.

Key words
***Platysternon megacephalum*, Hibernation, Body weight, Health, Environmental conditions.**

ABSTRACT

The big-headed turtle (*Platysternon megacephalum*) is endangered in the wild because of illegal trade, overharvesting and habitat destruction. Captive breeding is an effective protection against extinction, but safe overwintering is still technically difficult to captive big-headed turtles. Determining optimal hibernation conditions is important in resolving this problem. In this study, the effects of two important environmental conditions (water depth and availability of sand beds) on changes in body weight and health status in captive big-headed turtles before and after hibernation were evaluated. Results showed that providing a suitable sand bed during hibernation have a significant effect on turtle health ($P< 0.05$), but makes no significant contribution to body weight maintenance. An appropriate water depth (less than three times body height) is helpful for body weight maintenance ($P< 0.01$) when the ambient temperature is not cold enough for normal hibernation. These findings can provide technical guidance for safe overwintering of big-headed turtles in captivity, and will help facilitate conservation of this endangered freshwater turtle species.

INTRODUCTION

The big-headed turtle (*Platysternon megacephalum*) is found in China, Cambodia, Laos, Myanmar, Thailand, and Vietnam (Shen *et al*., 2010; van Dijk *et al*., 2011), but is currently endangered because of overharvesting, illegal trade, and habitat destruction. In order to control the illegal trade in big-headed turtles, this species is listed in CITES Appendix I. Guangdong is the biggest turtle trade centre in China (Gong *et al*., 2009), and several hundred live big-headed turtles of unknown origin have been seized from illegal markets in recent years. Since 2008, in order to protect the seized big-headed turtles, a plan of *ex situ* conservation with captive breeding has been carried out by the South China Institute of Endangered Animals. However, safely overwintering in captivity has been one of the major technical difficulties in this project, illness even mortality is higher during this period compared to the rest of the year. Therefore, optimizing the overwintering conditions for big-headed turtles is of significance in the success of the *ex situ* conservation project.

Turtles are ectothermic animals, relying on their environment for thermoregulation, and they use hibernation to survive the cold winter period (Gregory, 1982; Carey *et al*., 2003). Hibernation in the wild is a risky activity, with several threats to overwintering survival, including freezing, metabolic and respiratory acidosis, predation, energy store depletion, and immune depression. Selection of optimal hibernation sites can minimize the risks. Based on our field study, the big-headed turtles generally stay under water during hibernation, meanwhile, they prefer to fix themselves in a cave and bury themselves into sand beds under water, rather than put themselves in a spacious and bright place. Staying under water during hibernation

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has great benefits for a freshwater turtle species, because of the relatively small temperature variations in aquatic environments (Ultsch, 1989; Dartay *et al.*, 2017). However, oxygen depletion can be another problem for air-breathing ectotherms that hibernate underwater. They require either effective aquatic respiration if dissolved O_2 is available or the capacity for prolonged anaerobic metabolism if O_2 supplies are limiting. An optimal water depth may be important to balance the ambient temperature and oxygen content for hibernating turtles (Ultsch, 1985; Druzisky and Brainerd, 2001; Jackson and Ultsch, 2010). The cave or sand beds seems to have little contributions to the ambient temperature or oxygen content. Why the turtles prefer to fix themselves by a cave and sand beds during the hibernation? Is it necessary to provide such sand beds for *P. megacephalum* in captivity during the hibernation?

During hibernation, even though the hibernators have low levels of activities and depressed metabolism to avoid the excessive energy consumption, the body weight loss is inevitable (Derickson, 1976; Jackson and Ultsch, 2010), and the depressed metabolism can also affect the function of the innate and adaptive immune system responses in hibernators, making them more vulnerable to pathogens (Zapata *et al*., 1992; Luis and Hudson, 2006). In order to evaluate the effects of two ecological factors (water depth and sand bed) for a successful hibernation, the changes of body weight and health status of captive big-headed turtles before and after hibernation were investigated.

METHODS

Experimental animals and group setting

All of the experimental turtles were raised in a turtle conservation center, located in a nature reserve in Huizhou, Guangdong, China. The turtles were fed with fresh loaches, eels, mountain stream fishes, freshwater snails, river shrimps and mammalian liver once a day. A total of 48, 60 and 76 different individuals were observed in three winters (2011-2013), respectively. In winter, all experimental turtles were kept in a dim and quiet room, the range of light intensity in the bucket without shelter is 0-0.5 lx. Each of the turtles was kept in an individual bucket made of non-transparent plastic, with a diameter and height of 35 cm and 34 cm, respectively, during hibernation. The feed was stopped during the hibernation, and the hibernaculum was checked every day. The effects of water depth and substrate (sand bed) were investigated, and two levels were assigned for each factor.

Water used in the study was obtained from a local mountain stream, where *P. megacephalum* inhabit naturally. In order to test the effects of water depths, two different water depths were set each year for the duration of the experiment. In 2011, the water depth in level one was half the carapace height of the experimental turtles (0.5×, with an average water depth of 2.5 cm); for level two, the water just submerged the turtle's carapace (1×, with an average water depth of 5 cm). In 2012, water depths of 5 cm and 10 cm (1× and 2× the average carapace heights of the experimental turtles) were used. In 2013, water depths of 5 cm and 15 cm (1× and 3×) were used.

The sand beds used in the observation were also sourced from the turtles' natural habitat. Sand grain sizes ranged from 0.1-2 mm in diameter, and the material was soft enough for the turtles to bury themselves in. We supplied a sand bed with a depth of 4 cm underwater and used control groups without sand beds for comparison. All of the turtles were divided into 4 uniform groups, with no difference between groups with regard to sex ratio, average body weight or carapace length ($P > 0.05$).

Temperature measurements

In order to quantify environmental conditions during hibernation, air temperature inside of the hibernaculum were recorded every 2 h using a temperature data logger (AZ Instrument Corp., Taiwan).

Measurements of body weight

The body weights of the experimental turtles were measured using an electronic balance with a weighing accuracy of ± 0.1 g. Before weighing, turtles were cleaned carefully, and then towel-dried. The body weights of experimental turtles were measured at the beginning and at the end of hibernation.

Examination of the health status

The health status of the experimental animals was evaluated based on physical examination including body weight, appetite, eye and skin checks. Only healthy turtles were selected as experimental animals. After hibernation, the health status of the turtles was evaluated again. Skin diseases in the form of rots and eye diseases characterized by swollen eyelids were the most common problems after hibernation in captivity. Four levels of disease severity were defined: L0 (healthy), L1 (slight illness, for example, having a small amount of skin rot or eye disease), L2 (illness, for example, having skin diseases throughout the limbs, or serious eye disease), and L3 (serious illness, for example, serious skin and eye diseases). The healthy status of all experimental animal were determined subjectively by the three observers, and the final determination is by majority.

Data analysis

The SPSS 16.0 statistical package was used for statistical analysis. The body weight changes (g) were calculated as initial body weight (before hibernation) minus final body weight (after hibernation), and then ANCOVA (Analysis of Covariance) was used to evaluate the effects of water depth and sand bed, using the initial body weight as a covariant. Chi-square tests were used to evaluate the effects of the two factors on health status each year, with the level of significance set at $P< 0.05$.

RESULTS

Temperature conditions during hibernation

During our study, temperature conditions inside of the hibernaculum were recorded. The temperature ranges during the three winters were 11.5-20.5°C, and the average temperatures of the three winters were 15.1°C, 15.4°C and 17.4°C in 2011, 2012 and 2013, respectively. Data showing changes in temperatures during the three observation periods are displayed in Figure 1.

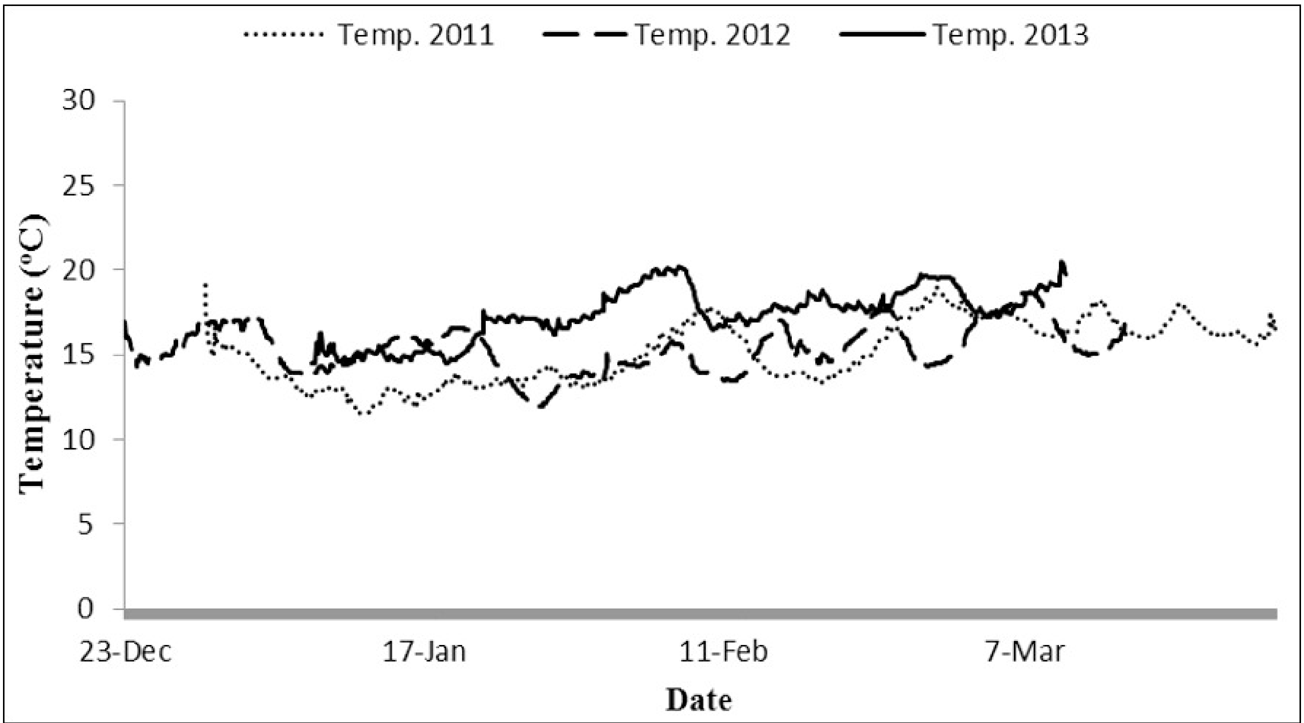

Fig. 1. Temperature records for overwintering of captive big-headed turtles in 2011, 2012 and 2013.

Table I.- Changes in body weight and health status for captive big-headed turtles (*Platysternon megacephalum*) before and after hibernation.

Year	Number	Days	Items	Body weight changes (g)				Health status			
				Min	Max	Mean	SD	L0	L1	L2	L3
2011	48	102	Value[a]	-45.1	1.5	-12.5	9.1	40	7	1	0
			Ratio[b]	-10.0	0.6	-4.1	2.6	83.3	14.6	2.1	0
2012	60	86	Value	-36.6	-0.6	-14.6	8.8	36	18	5	1
			Ratio	-9.7	-0.2	-4.9	2.2	60.0	30.0	8.3	1.7
2013	76	63	Value	-24.5	6.5	-8.2	6.9	39	28	7	2
			Ratio	-9.8	2.3	-3.0	2.7	51.3	36.8	9.2	2.6

[a], value of the body weight change means the absolute change in the turtles' body weights after hibernation; the value of the health status means the number of individuals at each health level; [b], ratio of the body weight change (%) is the absolute body weight change after hibernation divided by the initial body weight at the beginning of hibernation; the ratio of the health status (%) means the percentage of individuals at each disease level.

Changes in body weight and health status

Turtles on average lost 12.5 g (4.1%), 14.6 g (4.9%) and 8.2 g (3.0%) of body weight during hibernation in three years of observations. The maximum loss was 10% relative to initial body weight, but it is worth mentioning that some individuals gained weight during hibernation, with a maximum of 2.6% of initial body weight, suggesting that body weight may be affected by complex factors during hibernation. After hibernation, health statuses (L0 to L3) were assessed, and the results showed that 83.3%, 60.0% and 51.3% of experimental turtles were totally healthy after hibernation in the three years' respective observations, while 14.6% to 36.8% of individuals developed slight illness during hibernation (Table I).

Effects of sand bed and water depth on changes in body weight and health status

Based on data analysis, the results showed that: (1) providing suitable sand beds during hibernation can significantly improve turtle health (P=0.021, 0.007 and 0.004 in 2011, 2012 and 2013, respectively), but this makes no contribution to body weight maintenance; (2) appropriate water depth is helpful for body weight maintenance (less than twice the carapace height, P> 0.05, in 2011 and 2012). Turtles lose more body weight with increased water depths, as shown in Table II.

DISCUSSION

Hibernation has been documented in species from a wide range of taxa, and is thought to be an adaptation to cold environments and an energy-conserving strategy (Geiser, 2004). The major energy stores of reptiles and amphibians are glycogen and lipids; the reduction or depletion of lipids during hibernation is accompanied by decreases in glycogen stores, and the inadequate hibernaculum conditions may result in extra energy consumption (Derickson, 1976; Jackson and Ultsch, 2010). In this study, turtles lost 3.0-4.9% of their body weight on average during hibernation, and the turtles lose more body weight with an increase in water depth from 1× to 3× the turtle carapace height. We speculated the increasing body weight losing may be related with the environmental temperature. Species that hibernate submerged have to balance the relationship between temperature, energy metabolism and oxygen consumption (Jackson and Ultsch, 2010). If the ambient temperature is cold enough, and metabolism is extremely low, turtles can obtain sufficient oxygen by cutaneous gas exchange alone (Ultsch and Jackon, 1982; Reese *et al*., 2003; Jackson and Ultsch, 2010). When the ambient temperature is not cold enough, turtles will maintain a higher metabolic rate in which dissolved oxygen cannot meet the turtles' requirements, and they will have to move around to get oxygen (Meeks and Ultsch, 1990).

Table II.- Effects of sand bed and water depth on changes in body weight and health status for captive big-headed turtles (*Platysternon megacephalum*) before and after hibernation.

Conditions	Year	Levels	Body weight changes			Health status				
			LSM	SE	P value	L0	L1	L2	L3	P value
Sand bed	2011	-	-12.6	1.8	0.974	17	6	1	0	0.021*
		+	-12.5	1.8		23	1	0	0	
	2012	-	-15.0	1.3	0.635	13	12	4	1	0.007*
		+	-14.1	1.3		23	6	1	0	
	2013	-	-7.8	1.1	0.701	13	17	5	2	0.004*
		+	-8.4	1.1		26	11	2	0	
Water depth	2011	0.5×	-12.7	1.8	0.898	21	2	1	0	0.493
		1×	-12.4	1.8		19	5	0	0	
	2012	1×	-14.2	1.3	0.655	18	11	2	0	0.973
		2×	-15.0	1.3		18	7	3	1	
	2013	1×	-6.0	1.1	0.010*	19	14	2	1	0.649
		3×	-10.2	1.1		20	14	5	1	

Least-squares means (LSM) were used to determine the level of significance in pair-wise comparison, and pair-wise comparison was performed using Fisher's least significant difference (LSD) test, with the level of significance set at P< 0.05. Chi-square tests were used to evaluate the effects of the two factors on health status each year, with the level of significance set at P< 0.05.

Alternatively, the anoxic state requires a shift to anaerobic glycolysis, an inefficient pathway because of the low yield of ATP per glucose molecule, and one that disturbs acid-base balance due to the production of lactic acid (Jackson and Ultsch, 2010). The hibernaculum in this study is located in the south subtropical humid monsoon climate zone. The temperature inside the hibernaculum fluctuated from 10°C to 20°C during hibernation (Fig. 1), producing a high metabolic rate in the experimental animals. The turtle may need more activities to acquire oxygen in the water depth of 3×carapace height, and inducing more body weight loss at the same time.

Interestingly, we also observed that several turtles' body weights increased during hibernation, with a maximum increase of 2.6% of the initial body weight. Body weight is a comprehensive reflection of body composition, varying with many factors. For example, Bradford (1984) confirmed body weight increases during dormancy in the adult mountain yellow-legged frog (*Rana muscosa*) due to edema, even though fat content and the digestive tract were declining in mass (Bradford, 1984; Penney, 1987). In this study, we could not determine the tissue water content directly, so it is difficult to exclude the probability that water content changes may mask some of the energy store consumption. It would be helpful to find a noninvasive way to monitor the changes in turtles' energy stores directly, and thus evaluate energy consumption more accurately.

Besides the body weight losing, the hibernation may increase the risk of infections for the hibernators. Based on our observations, turtles are susceptible to infections in their limbs, paws, neck and eyes during hibernation, but providing suitable sand beds during hibernation may have significant positive effects on turtle health. In buckets with sand beds, turtles often kept quiet, and buried themselves in the sand bed, exposing only their heads. However, in buckets without sand beds, turtles were often agitated and hyperactive compared with the turtles hibernated with sand beds. It is proved that psychological factors have a huge impact on turtle behavior (Griffin, 1978). Providing sand beds during hibernation may increase the turtles' sense of security and decrease the damage to the immune system caused by stress responses. Evidence supports the notion that stress and emotional distress may relate to dysfunction and hypofunction in animals' immune systems (Solomon *et al*., 1974). In response to stressful environmental stimuli, glucocorticoids are released, which can result in beneficial short-term effects. However, when elevated glucocorticoid levels are long-lasting, as in chronic stress, glucocorticoids can have negative effects such as reproductive shutdown and decreased immunity (Reilly, 2010). Another negative influence of long-lasting glucocorticoid exposure may be decreased integrity of skin collagen. As the main component of skin, collagen has a key role in providing integrity and elasticity to this organ. Stress seems to affect the integrity of skin collagen through glucocorticoid-mediated processes that alter its synthesis and degradation (Kahan *et al*., 2009). The turtles overwintering without sand beds probably endure more stress responses, which further damage the animal's weakened immune systems and their skin integrity, causing infections in their limbs, paws, neck and eyes. In recent years, stress responses have increasingly been used as bio-monitors for potentially threatened populations, and chronic stress may be useful in predicting the survival of stressed populations (Reilly, 2010). For captive breeding animals, we suggest that reducing their stress responses as much as possible is important in maintaining health, and thus can be helpful in *ex situ* conservation projects.

The results of this study imply that supplying sand beds is helpful to keep turtles healthy during hibernation, and an appropriate water depth (less than three times of the body height) is helpful for body weight maintenance when the ambient temperature is not cold enough. These findings could provide technical guidance for safe overwintering of *P. megacephalum* in captivity, and may also have reference significance for other chelonian.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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