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Short Communication

B-ultrasonography: An Accurate and Noninvasive Method for Pregnancy Diagnosis in Pangolins

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ABSTRACT

Pregnancy diagnosis is critical to the management of captive breeding of wild animals, as accurate pregnancy diagnosis can improve the reproductive efficiency of captive animals. Pangolins do not show obvious morphological changes during pregnancy, which increases the difficulty of pregnancy diagnosis. In this study, pregnancies were diagnosed using B-ultrasonography in three Sunda pangolins (MJ-X2, MJ-X3, and MJ-X4) that had mated with males. B-ultrasonography revealed embryos in pangolins MJ-X2 and MJ-X3, but not in MJ-X4. Pangolins MJ-X2 and MJ-X3 gave birth to cubs 102 and 123 days, respectively, after B-ultrasound detection; pangolin MJ-X4 did not give birth to a cub. This study showed that B-ultrasound can accurately diagnose pregnancy in Sunda pangolins and detect the developmental status of their embryos and fetuses. As a method of pregnancy diagnosis, B-ultrasound is not only easy to operate, safe, and noninvasive, but also very intuitive and efficient compared with hormone detection methods. Efficient detection methods such as B-ultrasonography are critical to avoid housing already-pregnant pangolins together with males, which may mate with the females again or stress them, resulting in abortion. This approach also helps to conserve male reproductive resources, thereby improving the reproductive efficiency of captive Sunda pangolins. Effective and efficient captive breeding programs are critical for the conservation of Sunda pangolins and other pangolin species.

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SW and FZ conceived the project. YY wrote the manuscript.

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The pangolin (Pholidota: Manidae) is eutherian mammal (Heath and Vanderlip, 1988; Wu *et al*., 2005), and it is the only truly scaly mammal. Pangolins have become the most illegally trafficked mammals in the world, it is estimated that more than one million pangolins have been snatched from the wild between 2004 and 2013 due to the perceived medicinal properties of their scales and for their rich meat (IUCN, 2014). Pangolins are considered to be endangered, and the Sunda and Chinese pangolins were listed as critically endangered on the International Union for Conservation of Nature Red List (Challender *et al*., 2014a, 2014b). All species are listed in the Convention on International Trade in Endangered Species (Appendix I), and a zero export quota was established for wild-caught specimens of this species traded for primarily commercial purposes (CITES, 2019). Understanding the biology and ecology of pangolins is critical to formulate more effective species protection measures. However, pangolins are timid, secretive, and solitary nocturnal animals that are difficult to study in the wild. Pangolins smuggled from unknown sources and later confiscated can be used for *ex situ* conservation research. This approach enables the use of these valuable samples for biological and ecological research and avoids the wasting of resources and generation of negative ecological impacts *via* inappropriate release (Zhang *et al*., 2017; Zhang *et al*., 2019).

Pangolins are extremely difficult to maintain in captivity (Yang *et al*., 2007; Mohapatra and Panda, 2014; Zhang *et al*., 2017). Progress of pangolin husbandry development is hampered by the lack of scientific and standardized captive management protocols, especially regarding breeding (Challender, 2008). Pregnancy diagnosis in pangolins is one of the most important tasks during captive breeding. Accurate pregnancy diagnosis is critical for the timely separation of pregnant individuals from mating males, to prevent housing them together for long periods and/or repeated mating with males, which can result in stress or even abortion in pregnant females (PRB-SCNU unpublished data). In addition, the feed formula and intake can be adjusted appropriately to ensure adequate nutrition for the pregnant females. Accurate pregnancy diagnosis can also conserve valuable male resources and improve the reproductive efficiency of captive pangolins by avoiding unnecessary mating attempts. However, morphological changes are not obvious during pangolin pregnancy (Mohapatra and Panda, 2014; Zhang *et al*.,

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2016), except for weight gain (Chin *et al*., 2011; Zhang *et al*., 2016) and slight changes in the mammary glands in the later stage of pregnancy (van Ee, 1966; Heath and Vanderlip, 1988; Zhang *et al*., 2016). Therefore, pregnancy cannot be diagnosed accurately based on morphological changes alone, and it is necessary to look for more accurate methods of pregnancy diagnosis. Although Chin *et al*. (2011) diagnosed pregnancy and monitored the gestation period of pangolins *via* serum progesterone levels, this kind of method requires established criteria for diagnosis. Such criteria require long-term monitoring of multiple individuals, and the resulting indices may differ among species, which can be difficult to achieve, considering the endangered status of these animals. In addition, the process of blood drawing can have negative effects on pregnant pangolins and may lead to stress or even abortion. Therefore, the diagnosis of pangolin pregnancy using the maternal serum progesterone level is probably not the best choice.

The purpose of this paper is to introduce a noninvasive pregnancy diagnosis method (B-ultrasonography) for Sunda pangolins that can also be used for other pangolin species. Accurate pregnancy diagnosis can provide a scientific basis for captive breeding management of pangolins and is of great significance for *ex situ* pangolin conservation.

Materials and methods

The study animals were three wild female Sunda pangolins (MJ-X2, MJ-X3, and MJ-X4) rescued before May 1, 2012 (exact date unknown). They were housed at the Pangolins Research Base for Artificial Rescue and Captive Breeding of South China Normal University (PRB-SCNU) in Foshan city, namely the Foshan Huaman Pangolin Conservation Breeding Base. Housing conditions for the three Sunda pangolins were the same as those described by Zhang *et al*. (2015, 2017). The three female pangolins were housed together in the same enclosure with an adult male for mating between September 10, 2012, and November 15, 2012. Pangolins MJ-X2 and MJ-X3 gave birth to their young on March 22 and April 12, 2013, respectively (Zhang *et al*., 2015), whereas pangolin MJ-X4 had not given birth by the end of July 2013.

On December 10, 2012, an MSU2 all-digital mechanical fan scanning ultrasonic diagnostic apparatus (for veterinary use; Xuzhou Kaixin Electronic Instrument Co., Ltd., Xuzhou, China) was used to diagnose pregnancy in the three female pangolins. For the examination, the selected pangolin was placed on the operating table. One person controlled its two forelimbs, and another person held its two hindlimbs with both hands and forced them to the rear so that the animal's body was stretched out while it lay on the operating table. Then, the coupling agent was applied to the lower abdomen of the pangolin and the B-ultrasound probe was used for detection. Because pangolin abdomen hair is scarce, the animals did not need to be shaved. The whole process must be performed gently to avoid unnecessary roughness or stress, which could harm the potential fetus or even lead to abortion. As a result, the pangolin's body may not be fully stretched out during the procedure.

All experiments and animal housing were conducted in accordance with procedures approved by the Ethics Committee for Animal Research at South China Normal University, and the Guidelines for Animal Care established by the National Institute of Health. All efforts were taken to minimize pain and discomfort to the animal while conducting these experiments.

Results

The B-ultrasound detection results for the three female Sunda pangolins are shown in Figure 1. Hypoechoic pregnancy sacs with clear contours and hyperechoic tissue inside were observed in females MJ-X2 and MJ-X3 (a and b in Figs. 1A and B, respectively); that is, the embryos were clearly visible in the pregnancy sacs. For female MJ-X4, although an obvious hypoechoic entity (c in Fig. 1C) was observed, it contained no hyperechoic tissue; that is, no embryo was detected, which may be because the embryo was too small, or the female was not pregnant and the hypoechoic entity was her bladder. The reproductive information of the two pregnant pangolins is shown in Table I. The gestation length of the Sunda pangolins was 6 months (Zhang *et al*., 2015, 2017), and female MJ-X4 had not given birth by the end of July 2013. The reproductive status of the three female pangolins further demonstrates the accuracy of the pregnancy diagnosis results obtained using B-ultrasound.

In addition, based on the gestation length of Sunda pangolins and the delivery dates of the two pregnant female pangolins, these females were about 80 and 60 days pregnant, respectively, when they underwent B-ultrasound

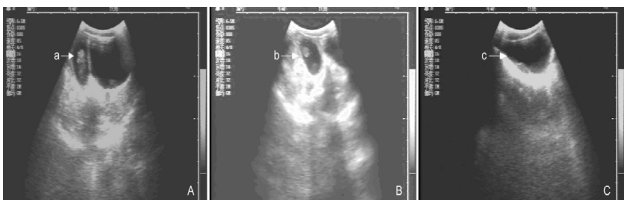

Fig. 1. B-ultrasonography images show that female pangolins MJ-X2 (picture A) and MJ-X3 (picture B) are pregnant, but MJ-X4 (picture C) not. Letters a and b show the pregnancy sacs with embryos on Dec 12, 2012. Letter c shows an obvious hypoechoic entity with no embryo.

Table I. The records of reproductive information of the two pregnant Sunda pangolins.

No.	Female ID	B-ultrasonic date	Mating date	Parturition date	Cub ID	Body weight of the cub
1	MJ-X2	Dec 10, 2012	Sep 10-Nov 15, 2012	Mar 22, 2013	MJ-X2b	90g (birth weight)
2	MJ-X3	Dec 10, 2012	Sep 10-Nov 15, 2012	Apr 12, 2013	MJ-X3b	285g (May 8, 2013)

examination. These results indicate that B-ultrasound can be used to diagnose pregnancy accurately in Sunda pangolins at the gestational timepoint of about 2 months. Furthermore, according to the development statuses of embryos MJ-X2b and MJ-X3b, as well as the pregnancy durations of their mothers, the embryo reaches the fetus stage within the first 2–3 months of pregnancy.

Discussion

Due to the increasing rarity and secretive, solitary, and primarily nocturnal nature of pangolins, combined with difficulties in maintaining them in captivity, little is known about the ecology and biology of these animals. Accurate pregnancy diagnosis and timely grasp pregnancy progress of female pangolin are not only conducive to scientific breeding management in pangolin conservation breeding, but also can provide basic information on pangolins. Pangolin body morphology shows no obvious changes during pregnancy (Mohapatra and Panda, 2014; Zhang *et al*., 2016), resulting in considerable difficulty with pregnancy diagnosis in captive pangolins. The lack of accurate pregnancy diagnosis methods may result in greater losses in captive breeding efforts. For example, at PRB-SCNU, due to the lack of pregnancy diagnosis methods, pregnant females were not separated from the male in time. Constant harassment from the male caused stress and eventually led to abortion (PRB-SCNU unpublished data). In another case, a female whose pregnancy was not detected was housed in the same enclosure as a male and mated with it (PRB-SCNU unpublished data). Yu *et al*. (2016) reported a significantly shorter gestation period for a Sunda pangolin than reported elsewhere for this species, which may also have been the result of mating during pregnancy. Although both of these females gave birth to one healthy cub each, the potential risks cannot be ignored. Therefore, as research informing pangolin rescue work progresses, the identification of an accurate, effective, and noninvasive method for pregnancy detection in captive animals is critical.

B-ultrasonography is a safe and harmless method for pregnancy diagnosis. It has been used widely for this purpose in many animals, including cattle (Jia *et al*., 2009), pigs (Ren *et al*., 2015; Dong *et al*., 2017), sheep (Wang *et al*., 2018), and alpacas (Xie *et al*., 2010). In this study, B-ultrasound accurately detected Sunda pangolin embryos at the gestational age of about 2 months, indicating that it can be used for pregnancy diagnosis in pangolins. Although Chin *et al*. (2011) reported the use of serum progesterone levels to monitor gestation length in Chinese pangolins, this method has some limitations. First, the biochemical characteristics of pregnant Chinese pangolins differ among individuals, and several samples were insufficient to establish an accurate index discriminating pregnant from non-pregnant status. Furthermore, progesterone levels in blood fluctuate to a certain extent, which leads to overlap of serum progesterone levels in pregnancy and non-pregnancy; namely, in the range of 10.9–18.5 ng/mL for Chinese pangolins (Chin *et al*., 2011). Levels measured in this range of overlap cannot be used to determine pregnancy status in pangolins. In addition, the gestation length of Chinese pangolins reported by Chin *et al*. (2011) is also controversial and may be an overestimation (Zhang *et al*., 2016). If so, the range of overlapping serum progesterone levels between pregnant and non-pregnant Chinese pangolins would be even larger. Finally, even if progesterone can be used to diagnose pangolin pregnancy status, large samples from different individuals of the same species and taken frequently during the same reproductive cycles from the same individuals are required to establish an accurate and reliable index. The large number of research samples needed and the potential for resulting injuries means that this method is not feasible for these precious animals. Additionally, because of interspecific differences, any index developed would not be applicable to other pangolin species. In comparison, B-ultrasonography is not only harmless to the test subject and not limited by the sample size; it can also be applied universally to the other seven pangolin species.

B-ultrasound examination has become routine for the monitoring of human fetal development and has been used widely to examine fetal development in pigs, alpacas, cows, yaks, and other animals (Hao *et al*., 2017; Xie *et al*., 2010; Wang, 2008). B-ultrasound examinations of two pregnant pangolins showed that the embryos, which had developed for about 60–80 days, had just reached the fetal stage. Research suggests that the development of fertilized pangolin eggs is relatively slow during the first half of fetal development, mainly during the organ formation period, whereas fetal development is rapid during the second half of gestation, when organs develop and mature. The results of this study suggest that frequent B-ultrasound monitoring of pregnant pangolins can be used to study the entire

fetal development process and all embryonic stages. By monitoring the development process in multiple fetuses, fetal health indicators for this species can be established without damage to the mothers or their fetuses, thereby providing basic data to evaluate the development and health status of pangolin fetuses in the future.

Conclusion

B-ultrasonography can be used as a noninvasive method for pregnancy diagnosis in Sunda pangolins. It can detect and study the embryonic development process in Sunda pangolins and to evaluate and diagnose fetal development and health status. Furthar, B-ultrasonography can be extended to pregnancy diagnosis and fetal development evaluation in other pangolin species.

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Statement of conflict of interest

The authors declare there is no conflict of interest.

References

Challender, D., 2008. Asian Pangolins: How behavioural research can contribute to their conservation. In: *Proceeding of the Workshop on Trade and Conservation of Pangolins Native to South and Southeast Asia* (eds. S. Pantel and S.Y. Chin) Singapore: Singapore Zoo, pp. 95-102.

Challender, D., Baillie, J., Ades, G., Kaspal, P., Chan, B., Khatiwada, A., Xu, L., Chin, S., KC, R., Nash, H. and Hsieh, H., 2014a. *Manis pentadactyla*. *The IUCN red list of threatened species*. 2014: e.T12764A45222544.

Challender, D., Nguyen Van, T., Shepherd, C., Krishnasamy, K., Wang, A., Lee, B., Panjang, E., Fletcher, L., Heng, S., Seah Han Ming, J., Olsson, A., Nguyen The Truong, A., Nguyen Van, Q. and Chung, Y., 2014b. *Manis javanica*. *The IUCN red list of threatened species* 2014: e.T12763A45222303.

Chin, S.C., Lien, C.Y., Chan, Y.T., Chen, C.L., Yang, Y.C. and Yeh L.S., 2011. *Zoo Biol*., **30**:1-11.

CITES. 2019. https://www.cites.org/eng/app/appendices.php (accessed 7 Jun 2019).

Dong, B., Wu, H., Li, L., Sun, H. and Peng, X., 2017. *Anim. Breed. Feed*, **12**: 4-5.

Hao, Z. and Yin, L., 2017. *Agric. Henan*, **12**: 47-48.

Heath, M.E. and Vanderlip, S.L., 1988. *Zoo Biol*., **7**: 293-312. https://doi.org/10.1002/zoo.1430070402

IUCN, 2014. *Eating pangolins to extinction*. https://www.iucn.org/content/eating-pangolins-extinction, (accessed 5 Jul 2019).

Jia, G., Wang, X., Wang, L., Lv, L. and Yu, L., 2009. *Shanghai J. Anim. Husband. Vet. Med*., **1**: 8-9.

Mohapatra, R.K. and Panda, S., 2014. *Folia Zool*., **63**:73-80. https://doi.org/10.25225/fozo.v63.i2.a4.2014

Ren, Q., Ren, H. and Huang, H., 2015. *Chinese Digest Anim. Husband. Vet*., **31**: 223.

van Ee, C.A., 1966. *Int. Zoo Yearb*., **6**: 163-164. https://doi.org/10.1111/j.1748-1090.1966.tb01734.x

Wang, Q., Xia, K. and Zhang, J., 2018. *Hubei J. Anim. Vet. Sci*., **39**: 9-12.

Wang, S., 2008. *Ultrasonographic assessment of embryonic and fetal development, and adult female genital system changes in the Yak.* M. Sc. thesis, Gansu Agricultural University, Lanzhou, China.

Wu, S.B., Ma, G.Z., Liao, Q.X. and Lu, K.H., 2005. *Studies of conservation biology on Chinese Pangolin* (Manis pentadactyla). China Forestry Publishing House, China.

Xie, J., He, J., He, X., Cheng, Z., You, R. and Dong, C., 2010. *J. Anhui agric. Sci*., **38**: 20722-20723.

Yang, C.W., Chen, S., Chang, C.-Y., Lin, M.F. Block, E., Lorentsen, R., Chin, J.S.C. and Dierenfeld, E.S., 2007. *Zoo Biol*., **26**: 223-230. https://doi.org/10.1002/zoo.20134

Yu, J., Peng, J., Liao, G., Jiang, F. and Ma, X., 2016. *J. Chongqing Normal Univ*. (*Nat. Sci*.), **33**: 33-36.

Zhang, F., Wu, S., Zou, C., Wang, Q., Li, S. and Sun, R., 2016. *Zoo Keys*, **618**: 129-144. https://doi.org/10.3897/zookeys.618.8886

Zhang, F., Yu, J., Wu, S., Li, S., Zou, C., Wang, Q. and Sun, R., 2017. *Zoo Biol*., **36**: 387-396. https://doi.org/10.1002/zoo.21388

Zhang, F., Wu, S., Yang, L., Zhang, L., Sun, R. and Li, S., 2015. *Folia Zool*., 64: 129–135. https://doi.org/10.25225/fozo.v64.i2.a6.2015

Zhang, F., Yu, Y., Wang, W., Xu, N. and Wu, S., 2019. *Oryx*, **53**: 411-412. https://doi.org/10.1017/S0030605319000358