



The intestinal microbiota of a Risso's dolphin (*Grampus griseus*): possible relationships with starvation raised by macro-plastic ingestion

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Abstract

Ingesting marine plastics is increasingly common in cetaceans, but little is known about their potential effects. Here, by utilizing 16S rRNA gene sequencing, we profiled the intestinal bacterial communities of a stranded Risso's dolphin (*Grampus griseus*) which died because of the ingestion of rubber gloves. In this study, we explored the potential relationships between starvation raised by plastic ingestion with the dolphin gut microbiota. Our results showed significant differences in bacterial diversity and composition among the different anatomical areas along the intestinal tract, which may be related to the intestinal emptying process under starvation. In addition, the intestinal bacterial composition of the Risso's dolphin showed both similarity and divergence to that of other toothed whales, suggesting potential roles of both host phylogeny and habitat shaping of the cetacean intestinal microbiome. Perhaps, the microbiota is reflecting a potentially disordered intestinal microbial profile caused by the ingestion of macro-plastics which led to starvation. Moreover, two operational taxonomic units (0.17% of the total reads) affiliated with *Actinobacillus* and *Acinetobacter lwoffii* were detected along the intestinal tract. These bacterial species may cause infections in immunocompromised dolphins which are malnourished. This preliminary study profiles the intestinal microbiota of a Risso's dolphin, and provides an additional understanding of the potential relationships between starvation raised by ingesting macro-plastics with cetacean gut microbiota.

Keywords Risso's dolphin · Gut microbiota · 16S rRNA gene · Macro-plastic ingestion

Introduction

Human-derived plastic debris in the ocean is ubiquitous and poses health risks to marine wildlife globally (Caruso et al. 2022; De Stephanis et al. 2013). Since cetaceans act as sentinel and indicator species for marine ecosystem assessment, there is no doubt that they are also threatened by

marine plastic pollution. The effects of plastics on cetaceans are closely correlated with their species-specific diving and feeding behavior (Eisfeld-Pierantonio et al. 2022). For example, large filter-feeding baleen whales tend to intake microplastics (<5 mm) when they engulf large amounts of water and mud, or from trophic transfer, while odontoceti predators are prone to ingest macro-plastics (25–1000 mm) when they capture plastic-shaped squids and fishes (Alava 2020; Alexiadou et al. 2019). According to the latest review, about 67.8% of cetacean species have been reported to interact with marine plastics, and the number of species has grown in the last decade (Eisfeld-Pierantonio et al. 2022). Specifically, compared to being entangled (34.4%; 31 species), a larger proportion of cetaceans was found to have ingested plastics (63.3%; 57 species), with macro-litter being the main issue for all. Even a tiny amount of ingested macro-plastic can have a huge impact on cetacean health, through blocking the gastrointestinal tract, followed by satiation, starvation, and malnutrition, and ultimately death can result. Even if the animal is able to survive a reduced

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quality of life, reproductive capacity can result (Eisfeld-Pierantonio et al. 2022; Gregory 1978, 1991). Moreover, the degradation of macro-plastics into micro-plastics can increase the ecotoxicological hazards, inducing the intestinal microbiota dysbiosis, in fish (Liao et al. 2022) and birds (Wang et al. 2021). The consequences of ingesting macro-plastics in cetaceans are becoming increasingly more serious as the ongoing COVID-19 pandemic has accelerated marine plastic pollution, like disposable face masks and gloves (Prata et al. 2020). Since the gut microbiota plays a critical role in host nutrition absorption, energy intake, and immune defense (Krajmalnik-Brown et al. 2012; Takiishi et al. 2017), recent research has highlighted the possible adverse effects of starvation and malnutrition on the gut microbiota (Million et al. 2017; Vera-Ponce de León et al. 2021). The absence of the normal intestinal flora could trigger the susceptibility of a host to bacterial infections (Nell et al. 2010). Therefore, gut microbial dysbiosis may aid in our understanding on how ingesting macro-plastics influences cetacean fitness. However, up to now, little is known about the potential relationships between plastic ingestion with the cetacean gut microbiota.

The Risso's dolphin (*Grampus griseus*), one of the largest toothed whales, is the sole species of the genus *Grampus* (family Delphinidae) and preys almost entirely on squids (Blanco et al. 2006). It has a cosmopolitan distribution, with a large range in water temperature (10–30 °C), water depth (under 10 m to deeper than 3800 m), and latitude (Jefferson et al. 2014; Kiszka et al. 2007). In China waters, the Risso's dolphin is widely distributed from the South China Sea and Taiwan waters, to the East China Sea and the Yellow/Bohai Sea. It is currently listed as the national second-class protected animal. Even though a recent retrospective study indicated numerous stranding events ($n=62$) of Risso's dolphins in China waters from 1950 to 2018, there is still a lack of understanding about their cause of death and potential threats to their health (Liu et al. 2022). One example is the case of a female adult Risso's dolphin which was stranded on the coast of the South China Sea near Zhanjiang City, Guangdong Province, in 2019 (Zeng et al. 2020). It was confirmed the dolphin ingested macro-plastics. However, as far as we know, the ingesting macro-plastics has not been described as a key issue for the conservation of the Risso's dolphin, due to insufficient information on the potential physical and pathogenic effects of macro-plastics on cetaceans.

In this study, to follow up a previous report (Zeng et al. 2020), we profiled the intestinal bacterial communities of a Risso's dolphin (*Grampus griseus*) stranded in South China Sea, and explored the potential relationships with starvation raised by macro-plastic ingestion.

Materials and methods

The Risso's dolphin and intestinal content sampling

On July 10, 2019, a female Risso's dolphin was stranded alive near Shimajiao waters in Xuwen County, Zhanjiang City, Guangdong Province, and died a half hour after being found (Zeng et al. 2020). The dolphin was then weighed, measured, and necropsied after death. As described by Zeng et al. (2020), the dolphin was emaciated, with extremely thin subcutaneous blubber; no apparent fatal traumas were detected, but two rubber gloves (> 30 cm) were found in the forestomach, and sands were detected in both the respiratory tract and esophagus. Moreover, the gastrointestinal tract was totally empty. It has been speculated that starvation and feebleness, following gastric blockage by rubber gloves, led to the live stranding and subsequent airway obstruction, which may have resulted in suffocation and finally death.

The intestinal tract was divided equally into three segments as the foregut, midgut, and hindgut. The luminal content from each intestinal segment was collected following the procedures outlined in a previous study (Wan et al. 2018). Three replicates from each intestinal segment were collected, resulting in a total of nine samples. All intestinal samples were stored in – 80 °C until DNA extraction.

DNA extraction, sequencing, reads processing, and statistical analysis

The metagenomic DNA from all the intestinal samples were extracted using the ZR fecal DNA kit (Zymo Research Incorporated, CA, USA), and then the concentration of extracted DNA was measured by a Nanodrop spectrophotometer. Qualified DNA products were amplified using the universal primers (338F: 5'-CCT AYG GGR BGC ASC AG-3' and 806R: 5'-GGA CTA CNN GGG TAT CTA AT-3'), targeting the V3–V4 regions of the bacterial 16S rRNA gene. PCR products from each sample were then combined for library construction using TruSeq Nano DNA LT Library Prep Kit, and sequenced by the Miseq Illumina platform (Majorbio Company, Shanghai, China) (2 × 250 bp paired ends). The raw sequencing reads were preprocessed as described in Wan et al. (2021). In brief, after removing sequencing reads of poor quality and chimeras, the 16S rRNA gene sequences were clustered into operational taxonomic units (OTUs) with USEARCH (Edgar 2010) on the Galaxy platform at 97% nucleotide identity. Taxonomic affiliations were then assigned through the RDP classifier with a threshold of 0.7 (<http://rdp.cme.msu.edu/>). All sequences were randomly resampled to the minimum depth of 27,623 sequences per sample. Phylogenetic trees were constructed using FastTree tools (Price et al. 2009). The alpha-diversity indices were

calculated using the Picante package in R, and compared between groups using the Wilcoxon rank sum test. Principal component analysis based on Bray–Curtis distances was further computed to evaluate the difference of microbial composition between groups. To identify biomarkers in each group, the linear discriminant analysis effect size (LEfSe, $p < 0.05$ and LDA score > 3.0) was analyzed online (<http://huttenhower.sph.harvard.edu/galaxy/>). To predict potential pathways from the 16S rRNA gene reads, the Functional Annotation of PROkaryotic TAXa (FAPROTAX) database was used (Louca et al. 2016).

Results and discussion

Overall microbial community structure and core microbiota

After rarefaction to 27,623 reads per sample, we obtained 62 OTUs in total, which is comparable with that from another stranded Risso's dolphin (67 OTUs) found in Korea waters (Kim et al. 2019). The dominate phyla were Firmicutes (83.03%), followed by Fusobacteria (12.50%) and Bacteroidetes (4.28%), with five rare phyla accounting for the remaining 0.19% of the total sequencing reads (Fig. 1a). The five most abundant genera were *Peptostreptococcus* (40.60%), *Paraclostridium* (31.36%), *Fusobacterium* (11.76%), *Vagococcus* (5.94%), and *Bacteroides* (4.27%), which together were 93.93% in relative abundance (Fig. 1b). The top four abundant genera were also present in all intestinal samples, constituting of the abundant “core microbiota” of the Risso's dolphin's intestinal bacterial community. This is quite distinct from the

microbial information of the Risso's dolphin stranded in Korea waters, which was predominately *Photobacterium* (89.4%) (Kim et al. 2019). The Risso's dolphin shared common gut microbial members associated with Firmicutes with a wide range of toothed whales, including the Chinese white dolphin (*Sousa chinensis*) (Wan et al. 2021), Yangtze finless porpoise (*N. a. asiaorientalis*) (Wan et al. 2016), bottlenose dolphin (*Tursiops truncatus*) (Wan et al. 2022), melon-headed whales (*Peponocephala electra*) (Bai et al. 2022), short-finned pilot whales (*Globicephala macrorhynchus*) (Bai et al. 2021), pygmy (*Kogia breviceps*), and dwarf (*K. sima*) sperm whales (Erwin et al. 2017), whereas at the genus level, the Risso's dolphin diverged from other toothed whales. For example, unlike *Halomonas*, *Photobacterium*, and *Cetobacterium*, detected as abundant taxa in the marine dolphins and porpoises (Wan et al. 2018, 2021, 2022), the Risso's dolphin contained higher proportions of *Vagococcus* (5.94%) and *Bacteroides* (4.27%) (Fig. 1b). This may suggest that host phylogeny and habitat can help shape diverse gut microbial communities in different cetacean species, or that it may reflect the disordered intestinal microbial profile of the Risso's dolphin which died of ingesting macro-plastics and starvation. Starvation may alter the morphology of intestinal epithelial cells and function of the gastrointestinal tract, and thus affect the microbiota colonized there (Okada et al. 2013). Therefore, physiological changes of the gastrointestinal tract during starvation may help explain how macro-plastic ingestion affect the microbial composition and diversity of the dolphin. However, the conclusion should be drawn with caution as only one individual was investigated in the present study.

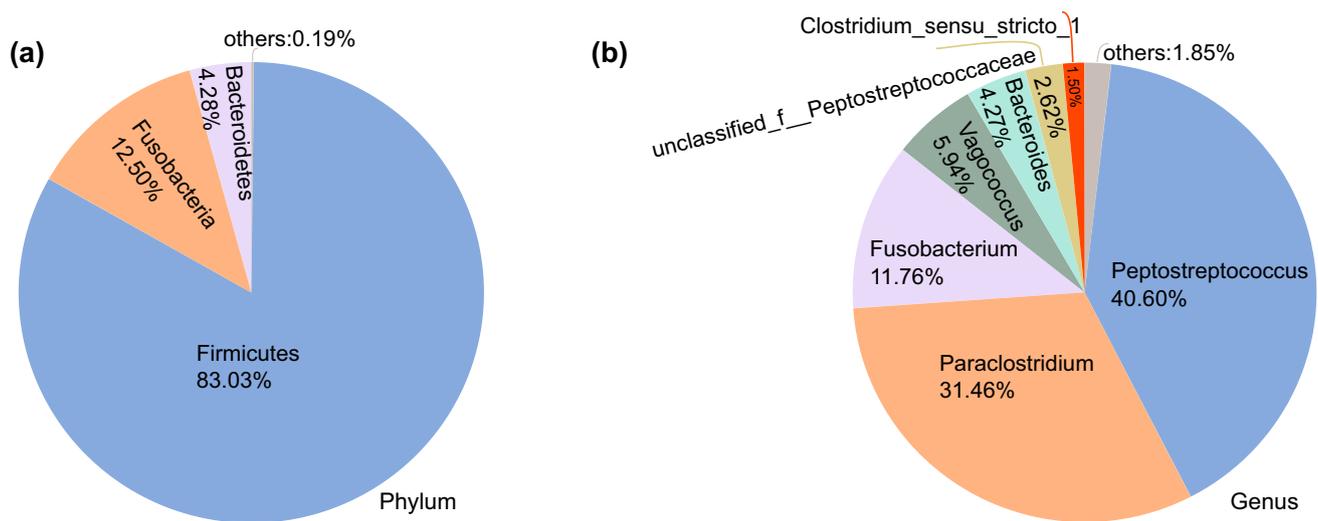


Fig. 1 Overall bacterial composition (relative abundance) of the intestinal samples. **a** Phylum level. **b** Genus level

Variations of bacterial diversity and composition

estimators (mean \pm standard deviation) of bacterial samples from three different intestinal regions

Region	Sobs	Chao 1	Shannon	ACE	Faith's PD
Foregut	34.00 \pm 2.00	37.01 \pm 2.21	1.46 \pm 0.05	41.67 \pm 4.82	3.61 \pm 0.12
Midgut	41.33 \pm 6.11	48.55 \pm 6.02	1.14 \pm 0.05	52.89 \pm 4.46	4.20 \pm 0.46
Hindgut	31.00 \pm 3.61	32.81 \pm 6.34	1.56 \pm 0.12	33.12 \pm 4.87	2.85 \pm 0.37

Sobs, the number of observed OTUs. Faith's PD, Faith's phylogenetic diversity

in different intestinal regions

In general, the alpha diversity, estimated by five indices (Table 1), was not significantly different among the three intestinal regions ($p > 0.05$), which could be due to the limited sample size. However, Sobs (the number of observed OTUs), Chao 1, ACE, and Faith's PD showed the highest values in the midgut, followed by the foregut and then the hindgut (Table 1). This is different with what was detected in a stranded Chinese white dolphin, which showed relatively higher diverse bacterial communities in the hindgut than the foregut and midgut (Wan et al. 2021). This difference between the Risso's dolphin and the Chinese white dolphin may reflect differences in their intestinal emptying processes. Specifically, it is speculated that the Risso's dolphin died not long after eating a prey item, after digesting her previous meal and emptying it to the midgut, leading to a higher midgut-associated bacterial diversity, whereas the Chinese white dolphin might have starved for a few days in the inland river, and emptied its chyme to the rectum. Therefore, the variation trend of the microbial diversity may change with host species or even with different nutritional statuses of the individuals. This may at least partly explain why there is currently no universal changing pattern of the intestinal microbial diversity of different cetacean species (Wan et al. 2018, 2021).

In agreement with previous findings (Wan et al. 2021), the bacterial community residing in the intestinal tract of the Risso's dolphin exhibited stratifications among the foregut, midgut, and hindgut regions (Fig. 2). These three regions harbored distinct bacterial genera communities (Fig. 2a, b). Typically, *Bacteroides* and *Porphyromonas*, both belonging to Bacteroidota, were significantly enriched in the foregut, while *Delftia* (belonging to Proteobacteria) and *Hathewayia* (belonging to Firmicutes) were abundant in the midgut (Fig. 2c, d) ($p < 0.05$). The hindgut, the proportion of *Rhizobiales* (belonging to Proteobacteria), was statistically higher than that in the other two regions (Fig. 2c, d) ($p < 0.05$). This intestinal region-specificity of bacterial communities may be correlated with the different micro-environments present in different intestinal regions. For example, oxygen tension decreases greatly from the small

tum of the large intestine favorable for enterohaemorrhagic *Escherichia coli* under anaerobic conditions (Woodward et al. 2019).

Functional potentials

Instead of exploring the general functional pathways of microbial communities along the intestinal tract, here we mainly focused on the potential pathogens, as well as plastic degradation-associated functions. In total, only 2 OTUs (0.17% of the total reads) affiliated with *Actinobacillus* (identity 99.53%) and *Acinetobacter lwoffii* (identity 100%), respectively, were potentially pathogenic by FAPROTAX analysis. The pathogenicity of these organisms has been experimentally verified in the literature. Specifically, *Actinobacillus* species (belonging to Pasteurellales) can cause actinomycosis, potent septicaemia, and fatal pneumonia in mammals (Rycroft and Garside 2000). And *A. lwoffii* (belonging to Pseudomonadales) can cause chronic gastritis (Rathinavelu et al. 2003). It is noticeable that the extremely low abundance of potential pathogens may be due to the low biomass of intestinal bacteria in the starving dolphin. Interestingly, in the present study, two rare OTUs, belonging to *Delftia tsuruhatensis* and *A. lwoffii*, respectively, were detected, both of which are related to plastic-degradation (Liang et al. 2008; Shigematsu et al. 2003). These bacterial species may be involved in the utilization of microplastics in the dolphin's gut, but further research is necessary to verify this possibility.

In conclusion, this study profiled the bacterial community composition and predicted functions along different intestinal regions of an adult Risso's dolphin which died of ingesting rubber gloves, leading to starvation and malnutrition. Even though more stranding cases are necessary to obtain reliable results, this report provides the first microbial identification in the different intestinal regions of the Risso's dolphin, which is associated with the potential relationships with starvation raised by ingesting macro-plastics. This result could serve as a reference for exploring the interactions between gut microbial dysbiosis and ingesting macro-plastics. This study highlights the dangers of the ingestion of plastics by cetaceans, especially since there is an increase in the disposable of plastics due to the COVID-19 pandemic.

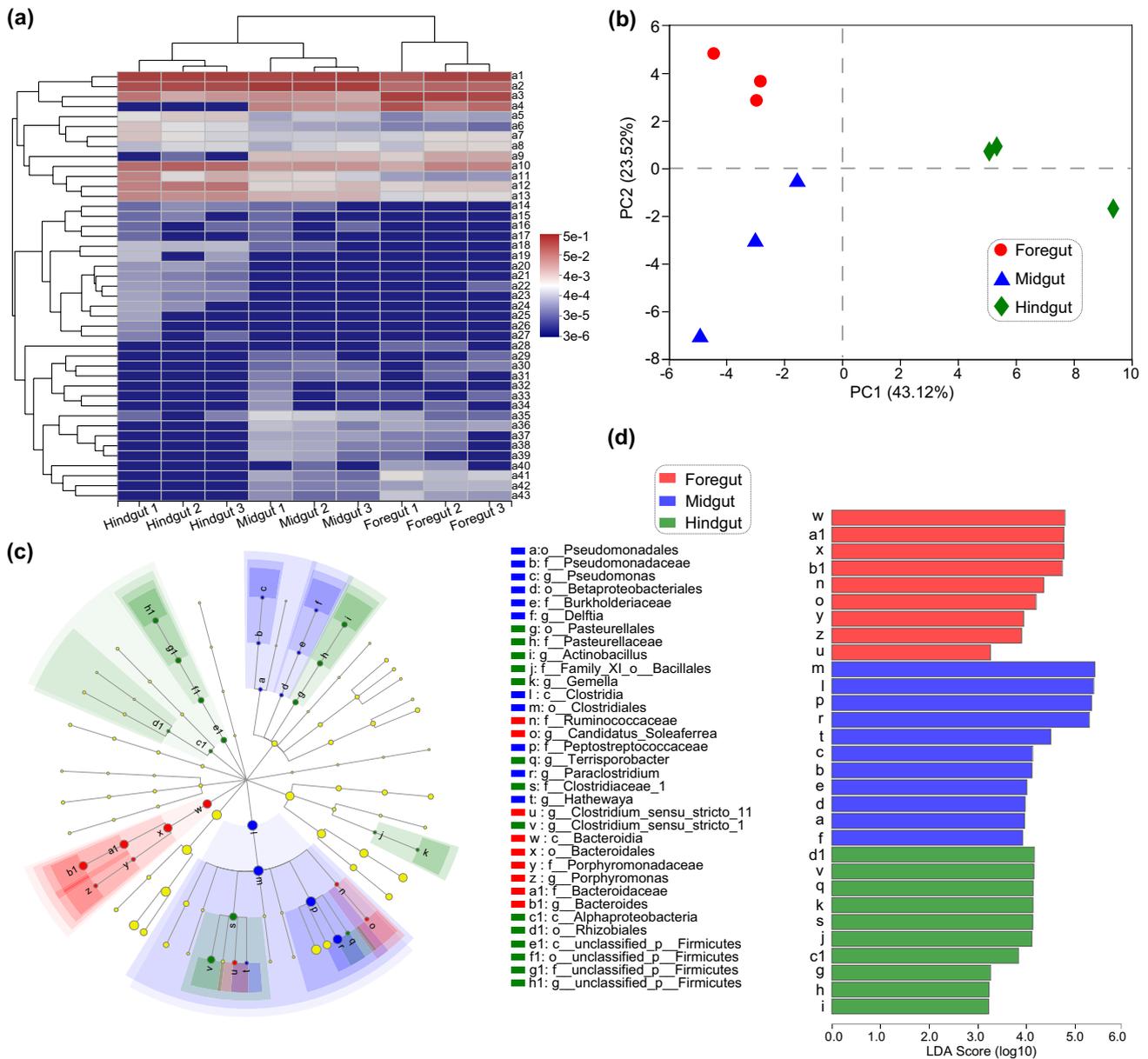


Fig. 2 The differential analysis of bacterial composition among three intestinal regions. **a** Community heatmap analysis of nine samples at the genus level. The IDs on the right denote genera in Appendix Table 1. **b** Principal component analysis (PCA) of all samples based on Bray–Curtis distances. **c** The taxonomic cladogram visualized by LEfSe analysis. Red, blue, and green areas represent foregut-, mid-

gut-, and hindgut-enriched bacterial taxa, respectively. **d** Linear discriminant analysis (LDA) scores (\log_{10}) obtained from the LEfSe analysis showed the biomarker bacteria taxa (LDA score > 3 with $p < 0.05$). The IDs on the left denote the genus information corresponding to the panel **c**

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10123-023-00355-z>.

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Author contribution Xiaoling Wan: methodology, software, funding acquisition, writing—original draft preparation. Jia Li: investigation, data curation. Mengxue Ao: methodology, formal analysis. Richard William McLaughlin: formal analysis, writing—review and editing. Fei Fan: writing—review and editing. Ding Wang: writing—review and editing. Jinsong Zheng: conceptualization, supervision, funding acquisition, writing—review and editing.

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Data availability All raw sequencing reads have been deposited in the NCBI database with accession number PRJNA876954.

Declarations

Ethics approval Carcass processing and sampling permission in this study had been authorized by the Bureau of Agriculture and Rural Affairs of Xuwen City, and were conducted in accordance with the Regulations of the People's Republic of China for the Implementation of Wild Aquatic Animal Protection (promulgated in 1993), adhering to all ethical guidelines and legal requirements in China.

Consent for publication All authors consent to the publication of this manuscript.

Competing interests The authors declare no competing interests.

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