The Zoo and Wildlife Nutrition Foundation Research Grant

Working Title: Investigating the nutrient requirements of the Sunda pangolin (*Manis javanica***)**.

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Purpose and Background Information: Sunda pangolins (*Manis javanica*) are one of the eight species of pangolins listed by the IUCN as threatened with extinction and one of the two species classified as critically endangered. Despite being listed in 2016 by CITES in its Appendix I, which prohibits international trade in wild-caught specimens or their body parts, pangolins are the most heavily poached and illegally traded species. Save Vietnam's Wildlife works with Cuc Phuong National Park to support the management and operation of the Carnivore and Pangolin Conservation Program (CPCP). CPCP and SVW work to rescue, rehabilitate and release carnivores and pangolins confiscated from the illegal wildlife trade; and on the development of global conservation breeding programs for threatened carnivores and pangolins. One of the greatest challenges to the successful rehabilitation and release of pangolins is nutrition and dietary husbandry. This includes challenges in hand-rearing pangopups. Nothing is known about the nutrient composition of pangolin milk.

According to the IUCN SSC Pangolin Specialist Group, over the last 160 years of record-keeping, more than 100 zoos and rescue centres have attempted captive management of pangolins with very limited success. The highest mortality rates are generally recorded in the first six months, with very few surviving even a few years in captivity. This failure is attributed in large part to the poor acceptance of captive diets (as they have adapted to a highly specialized diet in the wild including ants and termites), digestive problems, and stress. Many pangolins arrive emaciated and need specialized nutrition in order to adjust to captivity. Artificial diet formulations are poorly accepted.

In 2017, Taronga Conservation Society Australia (Taronga), began working with SVW on a captive pangolin diet, due to a diet formulated by their nutritionist, Michelle Shaw, for the captive management of short-beaked echidnas. A pilot study conducted at Taronga on short-beaked echidnas has shed light on the nutrition-related issues seen in this species and results suggest investigation into the nutrient requirements of species with similar feeding strategies, such as pangolins, may be critical to their captive management.

Short-beaked echidnas, like pangolins, are considered specialist carnivores but research being conducted by Taronga's Nutrition and Pathology departments is changing this view. A review of gastric pathology of captive and wild echidna necropsies from the Taronga Wildlife Hospital (1998-2015) revealed that 14/20 had gastritis lesions reminiscent of ruminal acidosis lesions typically caused by large amounts of highly digestible carbohydrate and low fibre levels in herbivore diets. All wild echidnas studied (n=7) had microscopically normal stomachs while all long-term captive echidnas (n=8) had moderate to marked gastritis. Microscopic comparison of stomach microfauna found that wild echidnas had a population of uniform coccoid bacteria lining the stomach. In contrast the inflamed stomachs of captive echidnas had mixed populations of rod bacteria and fungi. Microbiome

analysis confirms a difference in bacterial diversity in stomach and faecal samples between captive and wild echidnas. Captive echidnas had a higher percentage of bacteria within the Firmicutes phylum. This phylum includes families Lactobacillacea and Streptococcaceae. Bacteria within these two families are seen in the rumen contents of herbivores with ruminal acidosis.

Taronga formulated and introduced a new glucose-free diet (manufactured by Vetafarm) with added cellulose in 2014. Subsequently, necropsy of an aged echidna in 2016 revealed the first non-inflamed stomach in a captive echidna at Taronga Zoo in at least 20 years. It was hypothesized that the new diet improved digestive function and health in this individual. A study was undertaken to further compare wild and captive echidnas to improve understanding of nutrition and to make adjustments to the Vetafarm diet accordingly.

Stable isotope analysis (SIA) of echidna quills show the $\delta^{15}N$ value for zoo echidnas falls within the expected omnivore/carnivore range of their captive diet. As this profile is directly related to the diet of an animal, two species of termites and their termite mounds were also analysed for comparison with wild quills. The $\delta^{15}N$ value of wild echidnas is similar to termite mounds indicating that echidnas are assimilating nutrients from the mounds rather than the termites themselves. The $\delta^{15}N$ value in wild echidna quills suggests an herbivorous trophic position similar to deer. Taronga is working with TRAFFIC, Australian Nuclear Science and Technology Organisation and The University of New South Wales, to develop SIA techniques as a means to identify animals in import countries that are from captive breeding programs vs those illegally trapped for trafficking.

The data from stable isotopes, stomach pH, stomach morphology and pathology and microbiome confirm that the Short-beaked Echidna stomach functions similar to a herbivore rumen, utilizing resident bacteria to assist with digestion of food. There is some indication that this is also causing issues in other insectivores, such as pangolins, as similar stomach lesions have been reported and gastrointestinal issues are a major concern at SVW.

Taronga is currently investigating puggle stomach pathology. There is limited information regarding echidna milk, though what has been published indicates that carbohydrate concentration is very low and the main carbohydrates are oligosaccharides with little free lactose (Messer and Kerry, 1973). Milk oligosaccharides are thought to perform anti- and prebiotic functions, as opposed to providing nutrition directly to neonates (Sela and Mills, 2010). Echidna milk protein concentration was high (over 12%) as was Fe content at 33ppm (Griffiths et al., 1984), about ten times the concentration of cow and buffalo milk (Mondal et al., 2015). Iron is a limiting nutrient for bacterial growth. Echidna milk carbohydrate and Fe may perform important functions for establishing the appropriate microbiome in puggles. The milks of two other ant-eating mammals (aardvark and giant anteater) also are low in carbohydrate, and have a high proportion of energy from protein (aardvark = 41% of milk gross energy (White et al., 1985); giant anteater = 46% of milk gross energy (Power, 2015)). We hypothesize that pangolin milk will similarly be low in carbohydrate and high in protein, based both on diet and phylogeny (carnivore milk is generally high in protein and pangolins are a sister taxa to carnivores). Puggles and pangopups should be mother-reared whenever possible, but if not, appropriate milk replacers need to be developed. Current evidence suggest that these milk replacers should have little lactose or other simple carbohydrates, and provide substantial energy from protein, but solid data on milk composition is needed for both species, especially pangolins. This project will begin to set the nutrient parameters for milk replacer formulas for these species.

Methods: This study will include collecting samples from Sunda pangolins at SVW during their regular rescue and rehabilitation operations (see table below). Michelle Shaw will visit SVW prior to July 2018 to establish appropriate collection procedures based on resources (skills, time, storage facilities) at SVW. This visit will include performing post mortems on any stored deceased pangolins, collecting further samples for review by Taronga's pathologist, Dr. Lydia Tong, and organising all samples collected by SVW for shipment to the appropriate lab for analysis. Import/export permits will be arranged prior to the visit. Milk samples will be collected from echidnas in Australian zoos.

Funding Details: Michelle Shaw has been granted a $2000 fellowship through the Taronga Zoo Friends Fellowship Program to cover a 2-3 week visit to SVW prior to July 2018, pending Ministry approval. Researchers are applying for two Australian Research Council Linkage Grants next year to secure funding for a larger project that includes nutrition research and diet development over 3 years. One will focus on wildlife trafficking and developing a technique to differentiate between wild and captive bred animals. The second grant application will be focused on investigating the nutrient requirements of insectivores (particularly specialists like echidnas, pangolins, numbats etc) at different life stages to develop captive diets, including milk replacers. The projects use some of the same techniques so if we receive funding for one, we can do components of both projects. Each grant application will request ~$100,000 annually and has approval from the Taronga Foundation for $30,000 in cash to leverage these requests. These grants will not be available until late 2018, but will allow the project started during the fellowship and proposed in this application to be continued.

SAMPLE TYPE	TUBE / STORAGE TYPE	STORAGE	LAB/ANALYSIS
1) Saliva pH	N/A	Record Result	N/A
2) pH Gastric Contents	ASAP After Death OR During Intubation of Live Animal	Record Result	N/A
3) Gastric Contents: (Sterile / Near Sterile)	White Cryovial	Frozen (-80° or -20°)	Macquarie University
4) Milk 1-2ml (Sterile / Near Sterile)	White Cryovial	Frozen (-80° or -20°)	Smithsonian Institute
5) Faeces / Rectal Swab: (Swab if no Faeces Available)	Faeces or Swab tip in White Cryovial	Frozen (-80° or -20°)	Macquarie University
6) Hair Samples / 3x Scales (Clean / Dry / No Chemicals)	Sealed In Envelope	Room Temp.	UNSW and ANSTO
7)Deceased animals	Collect any samples from 1-6 and immediately freeze whole body.	Frozen (-80° or -20°)	Taronga

Funding Request: $1000USD is being requested from ZWNF. This funding will be used:

1) To create and ship a package for sample preparation to SVW. This package will include pH strips, sterile bags, sterile cryovials, sterile swabs and any other equipment and supplies required for collection of samples post mortem during the visit by Ms. Shaw.
2) To apply for import/export permits for samples from Vietnam to USA and Australia
3) To ship frozen milk samples from Australia (echidna) and Vietnam (pangolin) to the Smithsonian Institute and any remaining samples back to Australia for SIA, microbe analysis and histopathology.

It is expected that the funding will not cover all 3 items, but Taronga's Nutrition research budget will cover anything exceeding funding for this portion of the project before additional grants can be secured. Note: Dr. Michael Power will perform milk nutrient analyses (water, lipid, protein, carbohydrate, minerals, including Ca, P, K, Mg, Na, and Fe) for this project free of charge.

Dissemination Plan: Results of this portion of the study will primarily focus on the milk analysis being performed by Dr. Michael Power. Dr. Power and Ms. Shaw are members of various Nutrition groups internationally and will present findings at conferences held by one or more of these groups. If the number of milk samples are sufficient, the findings will be submitted to Zoo Biology for publication.

References

Brandis, K, Meagher, P., Tong, L., Shaw, M and Mazumder, D. 2017. Captive or Wild: Solving a critical problem in the wildlife trade: In: Australasian Wildlife Management Society Conference Proceedings.

Griffiths M, Green B, Leckie RMC, Messer M, Newgrain KW. 1984. Constituents of platypus and echidna milk, with particular reference to the fatty acid complement of the triglycerides. Aust J Biol Sci 37:323-329.

Messer M, Kerry KR. 1973. Milk carbohydrates of the platypus and echidna. Sci 180:201-203.

Mondal M, Pyne SK, Samanta G, Roy R. 2015. Studies on trace minerals in cow and buffalo milk in red latertic zone of West Bengal. Int J Bio-res Agril Sci 1:36-38.

Power ML. 2015. Nutrient composition of the milk of the giant anteater (*Myrmecophaga tridactyla*). 2015 Proceedings of the Nutrition Advisory Group.

Sela D., D. Mills. 2010. Nursing our microbiota: molecular linkages between bifidobacterial and milk oligosaccharides. Trends in Microbiology 18:298-307.

Shaw, M., Tong, L., Meagher, P., Brandis, K. and Mazumder, D. 2017. Short-beaked echidna (*Tachyglossus aculeatus*): Insectivorous herbivore? In: AZA Nutritional Advisory Group Annual Conference Proceedings.

Stannard, H.J., Bekkers, J.M., Old, J.M., McAllan, B.M., Shaw, M. 2017 Digestibility of a new diet for captive short-beaked echidnas (*Tachyglossus aculeatus*). Zoo Biology. DOI: 10.1002/zoo.21347

Tong, L.J., Shaw, M., Chong, D., Tobias, G., Hulst, F., Vinette Herrin, K. and Vogelnest, L. 2017 Gastritis in the short-beaked echidna (*Tachyglossus aculeatus*): parallels with ruminal acidosis in herbivores and implications for the understanding of echidna digestive physiology.In: AZA Nutritional Advisory Group Annual Conference Proceedings.

White J.M., G. Williams, J.H. Samour, P.J.J. Drury, P. Cheeseman. 1985. The composition of milk from captive aardvark (*Orycteropus afer*). Zoo Biology 4:245-251.