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測量圈養白鯨聲學活動及皮屑皮質醇作為識別緊迫因子方法

Measuring Acoustic Activity and Scrape Cortisol as A Method to Identify Stressors in Captive Beluga Whales (*Delphinapterus leucas*)

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本論文係黃頌晞君（R08629004）在國立臺灣大學獸醫學系研究所完成之碩士學位論文，於民國 110 年 6 月 25 日承下列考試委員審查通過及口試及格，特此證明

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中文摘要

近年來，在公眾對動物福利的關注由農場擴大至動物園及水族館，令福利科學的研究日益增長。在圈養動物中，壓力及其來源是福利問題的重點。由於壓力可以反映動物福利的不利影響，因此評估動物壓力的客觀方法將是必不可少的。使用行為和生理參數作為指標來定量評估動物福利，而不是簡單地定性評估，可以防止歸因偏差的發生，並且成為比單獨行為測量更有效的緊迫和動物福利指標。為了驗證這種方法，將測量台灣圈養白鯨 (*Delphinapterus leucas*) 聲學活動和皮屑皮質醇濃度作為識別緊迫因子方法。三頭圈養白鯨的聲學活動 (每小時 5 分鐘) 已由傳感器常規記錄，並使用音頻編輯軟件進行分析。根據已發表論文，白鯨的叫聲分為三大類，分別為口哨聲、脈沖聲和組合叫聲。每週於白鯨胸鰭及/或尾鰭非侵入性地刮取皮屑樣本一次。改良已發表論文中皮膚固醇類提取技術以提取皮質醇，並通過市售酶免疫測定法進行檢測。發現兩個採樣位置 (胸鰭及尾鰭) 的皮質醇濃度相似。通過使用評分方法分析從事件到皮屑皮質醇的時間滯後，注意到 68-72 天得分最高，說明白鯨的皮膚皮質醇可能代表 2 個月前出現的應激反應。應用時間滯後後，根據飼養和醫療記錄選擇事件的日期。三個具有高質量音檔的事件被用來進行壓力反應的分析。在每個事件中至少可觀察到一天的呼叫聲學活動減少。另外，雖要三頭白鯨的皮質醇濃度沒有顯示出統計學差異，但它們的皮質醇濃度各不相同。在不同個體中發現了對壓力的不同反應，表明動物的性格可以表現在皮屑皮質醇濃度的變化中。結果顯示，由於皮質醇濃度和聲學行為的變化可以反映環境和身體狀況的波動，因此測量皮屑皮質醇和記錄聲學活動是一種監測白鯨壓力水平的有價值方法。這項研究為圈養鯨類壓力和福利科學研究的提供基礎信息，可用於監測並隨後改善它們的生活質量，並將有助維護圈養鯨豚之動物福利並提高牠們的生活品質。

關鍵字：白鯨、聲學活動、皮質醇、緊迫、動物福利

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Abstract

With the broadening of focus from animals on farms to zoos and aquaria, the field of welfare science and public concern for animal welfare grows continuously. In captive animals, stress and its causes are topic of interest in welfare issues. Because stress could reflect the adverse effect in an animal's welfare, an objective method to assess animals' stress will be essential. Using both behavioral and physiological parameters as indicators to assess stress and animal welfare quantitatively, rather than simply qualitatively, should be more valid indicator of stress and animal welfare than behavioral measures alone and will prevent the occurrence of the bias cause by attribution. To validate this approach, acoustic activity and skin cortisol concentration were used to identify stressors in captive beluga whales (*Delphinapterus leucas*) in Taiwan. The acoustic activity (5 min per hour) of three captive beluga whales has been recorded by transducer routinely, and are analyzed using audio editing software. The beluga calls were separated into three main categories whistles, pulse, and combo calls based on previous studies. The scrape samples have been collected non-invasively once a week from all three animals' fluke and/ or flipper. Cortisol was extracted using a modified skin steroid extraction technique delineated in previous study, and detected via commercially available enzyme immunoassays. The cortisol concentration of the samples from two locations (flipper and fluke) could be similar. The time lag from the event to scrape cortisol was analyzed by

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using a scoring method. It is noticed that 68-72 day was with the highest score, indicating that the skin cortisol in beluga may present the stress response around 2 months ago. After applying the time lag, days of event were selected based on the husbandry and medical record. Three events, which had high quality recording, were selected for analyzing the stress response. The significant difference in the total number of calls of five days was observed in three analyzed events. A decrease of the total number of calls was also observed in at least a day of each event. The cortisol concentration among three beluga whales did not show a statistically difference, but their cortisol concentrations varied. Varied stress responses were found among individuals. This indicates that the animals' characteristics could be presented in the scrape cortisol concentration changes. The results presented here suggested that scrape cortisol measurement and acoustic recordings are a valuable method to monitor stress levels in beluga whales since the changes in the cortisol concentration and acoustic behavior could reflect the fluctuation of environment and body condition. This study may be the fundamental information of future studies on stress and welfare science for captive cetaceans, which can be used to monitor and subsequently improve their quality of life. This method would substantially contribute to cetacean husbandry and subsequently improve animals' quality of life.

Keywords: Beluga whales, Acoustic activities, Cortisol, Stress, Animal welfare

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Section1. Introduction

1.1 Behavioral and Physiological assessment in welfare

More than 700 million people visit zoos and aquaria worldwide each year, contributing to increased public awareness regarding animal welfare, and concurrent with an increase in welfare studies of captive animals. Maintaining a high standard of welfare in captive animals is not only important in ethical and legislative aspects (Salas *et al.*, 2016; Wolfensohn *et al.*, 2018), but also essential for establishing and maintaining good health in captive animals (WAZA, 2005; Salas *et al.*, 2018). When planning a welfare monitoring strategy, it is important that the full range of the animals' biological requirements and needs are considered (Wolfensohn *et al.*, 2018). Various components, which based on behavioral and physiological aspect, can be used to contribute to the objective assessment of animal welfare (von Fersen *et al.*, 2018; Wolfensohn *et al.*, 2018).

The goal for behavioral assessments is to ensure animals have a natural frequency and range of behaviors (Wolfensohn *et al.*, 2018). Although different recording methods can be used to contribute to the behavioral assessments, there should be aware that animal welfare is not being done from an anthropogenic viewpoint (Wolfensohn *et al.*, 2018). Besides the best know pathological forms of behavior-stereotypic behaviors, social behaviors, play behaviors and vocalization etc., can also be used in behavioral

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assessments (Mason and Latham, 2004; Boissy *et al*., 2007; Clegg *et al*., 2015). Previous study suggested acoustic activities can be used as measuring animal welfare in various animal species (Altmann, 1974; Norcross and Newman, 1999; Watts and Stookey, 2000; Hewson, 2004; Castellote and Fossa, 2006; Hemsworth *et al*., 2015)

The traditional measures of physiological parameters including changes of heart rate, respiration rate, blood pressure and/or temperature (Line *et al*., 1989; Line *et al*., 1989). While measuring these changes, it may require either restraint or implantation of telemetry devices, which may compound and confound stress measures. Measurement of leukocyte cell counts can also give an indication of the effect on the immune response. Animals under stress will have a significantly decrease in leukocyte response than animals that are not stressed (Honess *et al*., 2005). Another measurement is the detection of glucocorticoids (GCs, cortisol and corticosterone) in blood, faeces, urine, saliva, tears or hair (Champagne *et al*., 2018; Heimbürge *et al*., 2019). GCs is secreted by the adrenal gland under the activation of the hypothalamic–pituitary–adrenal (HPA) axis to exert a variety of physiological and behavioral effects (Möstl and Palme, 2002). The measurement of glucocorticoids is commonly used to monitor and indicate stress in zoo and wildlife medicine (Bayazit, 2009; Trumble *et al*., 2018). The concentrations of these glucocorticoids have been proposed as indicators of overall physiological state and

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provide vital information on the health and resilience of a population (Kershaw and Hall, 2016).

1.2 Stress and welfare

The five domains model, which was originally formulated in 1994 (Mellor and Reid, 1994), provide the fundamental assessment for animal welfare (Mellor *et al*., 2020). The five freedoms include freedom from hunger and thirst, freedom from discomfort, freedom from pain, injury and disease, freedom to express normal behavior, and freedom from fear and distress. In captive animals, stress and its causes are topic of interest in welfare issues (Waples and Gales, 2002). It is widely accepted that stress plays an important role in overall health of animals. In many cases, the physiological stress response is an adaptive feature to natural stressors. However, chronic and/or severe stress would lead to a compromised health, increase captive animals' susceptibility to diseases and lead to poor welfare (Veissier and Boissy, 2007; National Research Council, 2008; Golbidi *et al*., 2015; Lee *et al*., 2018).

The relationship between welfare and stress has been studied in the past 40 years. Moberg (Moberg, 1987) described stress could cause development of pre-pathological and this is the most defensible measure of stress affecting an animal's well-being. It is suggested that the welfare will become problematic when the vertebrates loose grip on their life conditions and the stress symptoms appear (Wiepkema and Koolhaas, 1993). A

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wider view gave an idea that, all biological responses can represent the state of welfare and the development of pathology as a manifestation of excessive stress which is also a poor welfare status (Broom and Johnson, 2019). It has been believed that a stressor that causes a disruption in the homeostatic will cause measurable behavioral and physiological changes that may impair the animal's welfare (National Research Council, 2008). Stress and associated health problems are a recognized concern for captive dolphins (reviewed in Waples and Gales, 2002). Managing stress and proper medical treatment and husbandry would be a prime importance in captive animal care. Identifying stressors in captive environment could be important for managing stress in captive cetaceans, which facilitating the maintenance of good welfare.

1.3 Stress and welfare assessment in captive cetaceans

Although cetaceans have been maintained in captive environment for over 150 years, there are few to no established methods to quantify and address captive cetacean stress and welfare (Clegg *et al*., 2015, 2021). When comparing occur of welfare articles between marine and terrestrial mammals, marines seems to be at least two decades later than terrestrials (Clegg *et al*., 2021). To the best of our knowledge, published empirical articles about captive cetacean stress and welfare are limited to four studies on bottlenose dolphins (*Tursiops truncatus*) (Waples and Gales, 2002; Ugaz *et al*., 2013; Monreal-

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Pawlowsky *et al*., 2017; Clegg *et al*., 2019) and one evaluation of beluga whales (*Delphinapterus leucas*) (Castellote and Fossa, 2006).

One bottlenose dolphins study used single physiological parameter, salivary cortisol concentration, to analyze the stress response after a construction (Monreal-Pawlowsky *et al*., 2017). Three bottlenose dolphins' studies used behavioral and physiological parameters to study using stress as a metric for welfare. A previous study analyzed the correlation between behavioral indices and physiological measures (include basic blood parameters and postmortem findings) of stress and health. Then it suggested the behavioral (observation) records can be an important early indicator of health problem (Waples and Gales, 2002). Another research suggested the design of pools may influence the welfare of dolphins by comparing behavior and salivary cortisol concentration in dolphins which were in open and closed facilities (Ugaz *et al*., 2013). Other researchers compare the willingness to participate (Wtp) in the training sessions and the dolphins' health state (health and appetite). It found that when the dolphin was sick, the Wtp would decrease significantly. Thus, Wtp was suggested to be a potential behavioral measure and an indicator of early changes in dolphins' health state (Clegg *et al*., 2019). In beluga whales, acoustic activities was suggested to be an indicator to evaluate the welfare by comparing the acoustic activities before and after transportation and the introduction of harbor seals (*Phoca vitulina*) (Castellote and Fossa, 2006).

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In 2015, the first comprehensive protocol with 36 measures quantifying the welfare of bottlenose dolphins in captivity was presented (Clegg *et al.*, 2015). With the standardized welfare assessment tool, that protocol produces a critical step to improve marine mammal husbandry. However, as this protocol was developed in a semi-open seawater facility, this may limit the efficacy of using the assessments in other captive environment (e.g. aquaria). Also, although this assessment is objective, there may be limitation about the conclusions made about dolphin welfare as the research is limited. For example, stereotypic behavior was an indicator of the abnormal behavior which may conclude the animal was in poor welfare. But some research suggested that animals exhibiting stereotypies as a coping mechanism may have better welfare than the non-stereotyping animals (Runshen and Mason, 2006).

1.4 Acoustic activities in beluga whales

Beluga whales are widely distributed odontocete cetaceans in the Arctic. Beluga whales belongs to the Monodontidae family, which is included together with the Delphinidae and Phocoenidae families. Due to its high acoustic activities and rich vocal repertory, beluga whales have earned the nickname 'marine sea canary' (Jefferson *et al.*, 2015). Their vocalization behaviors have been studied in captive and free-ranging environments (Belikov and Bel'Kovich, 2007; Vergara and Barrett-Lennard, 2008). Also, their calls have been widely described and classified (Chmelnitsky and Ferguson, 2012;

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Panova *et al*., 2012; Garland *et al*., 2015). The previous studies suggested that there are a number of similarities in the call types, but also presence of novel call types among different locations, suggesting the geographic differences among different populations (Karlsen *et al*., 2002). When beluga whales have a high acoustic activities and rich vocal repertory, it will be convenient to use vocalization as the behavioral assessment. Though the audio editing software, the recording can be easily quantify based on the duration, rate and frequency. Vocal behaviors have been used as an indicator of welfare in terrestrial captive mammals(Norcross and Newman, 1999), but little is known about the application of marine mammals. Previous studies showed that the acoustic activities of beluga whales would decrease after transportation and introduction of harbor seals (Castellote and Fossa, 2006). Thus, they suggested beluga whales' acoustic behavior is very sensitive to environmental stressors and the underwater acoustic monitoring is a potential method to evaluate welfare in beluga whales.

1.5 Cortisol studies in cetaceans

Although there are serval different sources of sample, which include blood (e.g., in bottlenose dolphins (Champagne *et al*., 2017, 2018) and beluga whales (Loseto *et al*., 2017)), feces (e.g., in bottlenose dolphins (Champagne *et al*., 2017, 2018) and right whales, *Eubalaena glacialis* (Rolland *et al*., 2017), blow (e.g., in beluga whales (Thompson *et al*., 2014)), saliva (e.g., in bottlenose dolphins (Monreal-Pawlowsky *et al*.,

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2017), skin (e.g., in harbor porpoises, *Phocoena phocoena* (Bechshoft *et al*., 2015), and bottlenose dolphins (Bechshoft *et al*., 2020)) and blubber (e.g., in short beaked common dolphins, *Delphinus delphis* (Kellar *et al*., 2015),and beluga whales (Trana *et al*., 2016; Loseto *et al*., 2017)). In cetacean cortisol researches, cortisol concentration in different sample represents different stress. The cortisol concentration in blood samples was suggested to reflect the acute stress level (Champagne *et al*., 2017, 2018; Loseto *et al*., 2017), while skin, blubber and baleen are suggested to reflect cortisol status over multiples weeks to years. In previous studies, saliva samples were used to investigate the welfare of captive bottlenose dolphins in different facilities (Monreal-Pawlowsky *et al*., 2017). In addition, both fecal and blow samples have been suggested to be non-invasive biological sampling sources for studying the stress load of cetaceans (Thompson *et al*., 2014; Champagne *et al*., 2018). Moreover, fecal samples have been used to study the impact of anthropogenic activities on cetaceans (Rolland *et al*., 2017). Skin and blubber cortisol levels have been proposed as potential candidates for long-term stress monitoring in stranded and free-ranging cetaceans (Bechshoft *et al*., 2015; Kellar *et al*., 2015; Loseto *et al*., 2017). Moreover, blubber samples were used to investigate the relation to cause of death (stranded or bycaught) in cetaceans (Kellar *et al*., 2015). In a long-term welfare assessment, it is important that sampling should not cause an adverse effect on welfare. Therefore, in the current study, we planned to use a new matric: scrape sample, which is

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the outermost part of the skin, mainly consisting of epidermal cells, as a tool to investigate the welfare and stress in captive beluga whales.

1.6 Cortisol detection methods

Gas chromatography–tandem mass spectrometry (GC-MS) was used to detect cortisol in cetaceans (Bechshoft *et al.*, 2015). This technique can provide researchers with an accurate cortisol concentration or each sample, but it is costly. Radioimmunoassay (RIA), which provides an accurate measurement with high sensitivity, is another method used to detect cortisol in skin samples from cetaceans (Houser *et al.*, 2010). However, radioactive compounds should be handled carefully to prevent hazards to the handler; moreover, a scintillation counter is required. Enzyme immunoassay (EIA) is another option for detecting cortisol in cetacean blubber samples (Kellar *et al.*, 2015). EIA is relatively easy to perform and the equipment for it is inexpensive and widely available. Although EIA is assumed to be less sensitive than HPLC-MS and RIA, Kellar et al. (Kellar *et al.*, 2015) successfully measured blubber cortisol concentration and studied the general cause of death using EIA. Meanwhile, the manufacturer mentioned that the EIA limit of detection is 45.4 pg/mL, which is supposed to be able to detect scrape cortisol according to the skin cortisol concentration in a previous study (Bechshoft *et al.*, 2015).

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1.7 Aim of study

Although GCs are commonly used markers for evaluating stress in animals (Möstl and Palme, 2002; Sheriff *et al*., 2011; Ovejero *et al*., 2013; Hadinger *et al*., 2015; Monreal-Pawlowsky *et al*., 2017; Heimbürge *et al*., 2019), it is believed that no single biological parameter can adequately inform on a stressful condition and no single stress response is present in all stress-related situations (National Research Council, 2008). Using different assessment to identify stressors could interpreted as a more valid indicator of stress than GCs alone Thus, it is recommended that using two objective and non-invasive indicators; behavioral (such as acoustic activities) and physiological (such as cortisol concentrations); could provide a comprehensive evaluation to investigate the stressor in captive beluga whales. Published empirical articles about captive beluga whales stress and welfare assessment are limited one evaluation (Castellote and Fossa, 2006). The current research represents the first effort use multiple assessments to identify the cause of stress of beluga whales in captivity. It also contributes to better understanding of cetaceans' health, care and management, as well as support informed public awareness of marine mammals in captivity.

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Section2. Materials and Methods

2.1 Scrape samples collection

Three beluga whales were recruited for this study, including an adult female (A) and two adult males (B and C). These belugas were acquired offshore of Russia at 3 y of age in 2002 They were then housed in indoor pools with 17 to 18 °C filtered and ozone sterilized natural sea water at the National Museum of Marine Biology and Aquarium (NMMBA). The lighting of the facility was controlled to provide 10h of daylight daily.

Scrape samples, the outermost part of the skin and mainly consisting of epidermal cells with high water content (Figure. 1), were collected from three captive beluga whales, once a week since March 2017. The protocol has been reviewed and approved by IACUC of NMMBA and Ocean Affairs Council (Approval number 1070003656). The beluga whales that participated in this study had received pre-sampling training in order to prevent potential stress during sample collection. The animals were also trained to emerge from the water and lift up their flippers and flukes voluntarily. A tongue depressor was run along the animals' left flipper or fluke multiple times and collected the epidermal cells in a tube. The sampling location for each animal should be the same. In the early stage of the experiment, collecting samples from fluke and flipper could provide enough samples while the body could not. Therefore, in the routine monitoring, sample collected

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from beluga A was collected from dorsal and/or ventral side fluke and beluga B and C from the fluke (Figure. 2). In the flipper-fluke comparison experiment, seven sets of scrape samples from flipper and fluke were collected on the same day for analyze the variation between different sampling locations. After collecting the samples, centrifuged at 10,000rcf for 3 minutes (min) and the supernatant was removed.

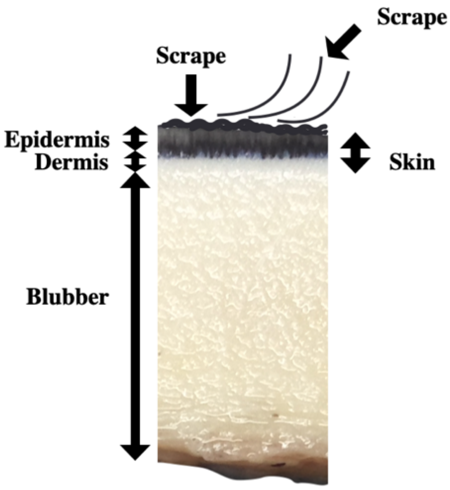

Figure. 1 Cross-section of cetacean skin.

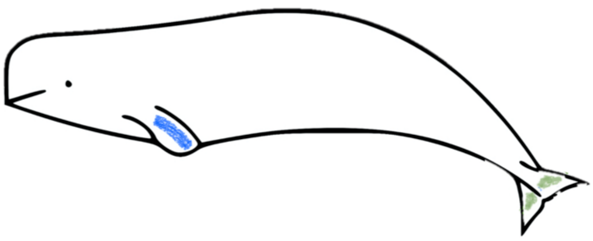

Figure. 2 Sampling location of routine monitor in beluga whales. Blue: sampling location of beluga B and C in routine monitoring. Green: sampling location of beluga A.

2.2 Hormone extraction

The method of extracting steroid hormones from the skin as described here is modified from the work of Kellar (Kellar *et al*., 2015). Glass beads for homogenization

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and the grinding media (glass beads) remained in the tube when adding the ethanol: acetone solvent. Approximately 0.08-0.15g of scrape sample were homogenized by vortexing with 1,000μL ethanol and 0.36g of 2 mm glass beads for 30 min. Then the mixture was centrifuged at 3000 rcf for 10 min. The homogenate/ glass beads solution was combined with 2 mL of 4:1 ethanol: acetone and vortexed for 10 min and centrifuged at 5000 rpm for 10 min. The supernatant was transferred into a new tube and evaporated by a stream of N2 vapor. Two mL of diethyl ether was added to the evaporated contents, vortexed, and centrifuged at 3000 rcf for 15 min. The supernatant was collected and evaporated, and the residue was resuspended in 1500 μL of acetonitrile, vortexed, and 1500 μL of hexane added to the mixture. After the solution was vortexed and centrifuged again, the hexane layer was removed and the process was repeated with another 1500 μL of hexane. The final acetonitrile layer was collected and evaporated. The final residue was centrifuged at 2500 rcf rpm for 5 min and stored at -20°C.

2.3 Cortisol detection

To prepare the samples for the enzyme immunoassay (EIA), the residue was suspended in 150 μL of 1M phosphate buffered saline and then was vortexed for 15 min. A commercial enzyme immunoassay was used to quantify concentrations of cortisol (#K003-H1, Arbor Assays, Ann Arbor, MI, USA). The manufacturer's protocols were followed exactly for the detection of cortisol.

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The extraction efficiency using spiked sample was determined as described in Kellar et al., (Kellar *et al.*, 2015) 320pg of cortisol were spiked with a known cortisol concentration sample. The extraction efficiency was calculated by using this equation, $E = Conc_0 - Conc_K / 320$. E is the extraction efficiency. $Conc_0$ is the concentration of the sample from EIA kit (pg/ml). $Conc_K$ is the concentration of the known sample. To calculate the final hormone concentration of each sample, use the following equation, $Conc_F = (Conc_1 * V_{PBS})/(1000*M*E)$. $Conc_F$ and $Conc_1$ are calibrated concentration of each sample(ng/g) and concentration of the sample obtained from EIA kit (pg/ml), respectively. V_{PBS} is the volume of PBS (ml) that used to suspend the sample, M is the wet weight (g) of each sample and E is the efficiency.

2.4 Parallelism and matrix effects analyses

Two additional quality control assessments were performed to gauge the performance of using the scraped extracts with the cortisol EIA kit. Parallelism was tested with the assay of six serial dilutions of scraped extract run along with known dose cortisol standards to determine whether the linear decrease in measured values of the scraped extracts was parallel to the standard curve, an indication that the assay is measuring the same antigens in scrape as in the standards. The scraped extracts from stranded pygmy killer whale were used to represent the result of parallelism. The sample concentration was made by a 2-fold serial dilution. Each dilution was run once, and the resulting curve

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of the detection metric (optical density of the sample / optical density when no sample is added(B/B0)) as a function of the dilution state was then compared to the standard curve using a F-test.

The second assessment examined the potential matrix interference, the effect of scrape extract itself on the measurement of cortisol. A standard solution was spiked with phosphate-buffered saline or/and a set of serial dilutions of a pooled sample composed of sixteen beluga scrape cortisol extract to make a final equivalent volume of 150μL. Each of the four serial dilutions was run in duplicate. The concentration of cortisol contributed from the pooled sample was subtracted from each sample-spiked measurement so its contribution would be factored out of the assessment.

2.5 Husbandry and medical records collection

The husbandry and medical record, which includes the daily food intake of each beluga whale, daily physiological assessment, symptoms, assessment, and treatment where health problems occurred and the environmental changes: water temperature, the depth of the water and construction, was collected since January 2017.

2.6 Scoring Method

The incorporation of cortisol (and other hormones) is believed to be due to skin renewal, which can deliver hormones to the margin of the skin. Therefore, there would be a time lag between the date of event and the present of cortisol signal in scrape. It is

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assumed that the time lag would be either 28-32, 38-42, 48-52, 58-62 or 68-72 days. The cortisol concentration greater than Q3 in the box plot was considered as a peak. Three, 6 and 4 peaks were identified in beluga whale A, B and C respectively. The comparison between cortisol concentration peaks and husbandry and medical records was performed to find out the most matching dates. The time lag was estimated by a scoring method mentioned below. When the cortisol peak can match with the husbandry and medical record, one score would be given. The maximum score for each cortisol peak is one. If there was more than one event can match the peak, only one score could be given. However, when the event happened more than once, and only a peak was being observed, that event could not get a score. The days that have maximum scored would be applied.

2.7 Acoustic activities

In order to record the acoustic activities of three beluga whales, the transducer was placed next to the gate (Figure. 3) which was kept enclosed to protect the transducer from damage caused by beluga whales. The acoustic activities (5 min per hour) of three captive beluga whales have been recorded (SM4 with hydrophone, Wildlife Acousticm, US) from March 2017 to March 2018. After applying the time lag, days of event were selected based on the husbandry and medical record. Five minutes acoustic activities which include the day of events and two days before and after the event were analyzed by listening and visually scrolling through spectrograms in audio editing software

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(Kaleidoscope, Wildlife Acoustics). The recording of 10:00 am, which was not a feeding session or the interaction with the keepers or within half an hour of any husbandry procedure, was selected as the representative of each day.

According to the previously published studies, beluga calls were separated into three main categories((1) whistles（2) pulse, and (3) combo calls) based on the acoustic parameters and variations in vocalization spectral contours (Belikov and Bel'Kovich, 2007; Chmelnitsky and Ferguson, 2012; Panova *et al*., 2012). The total number of calls and the pattern of calls were identified by two experienced observers.

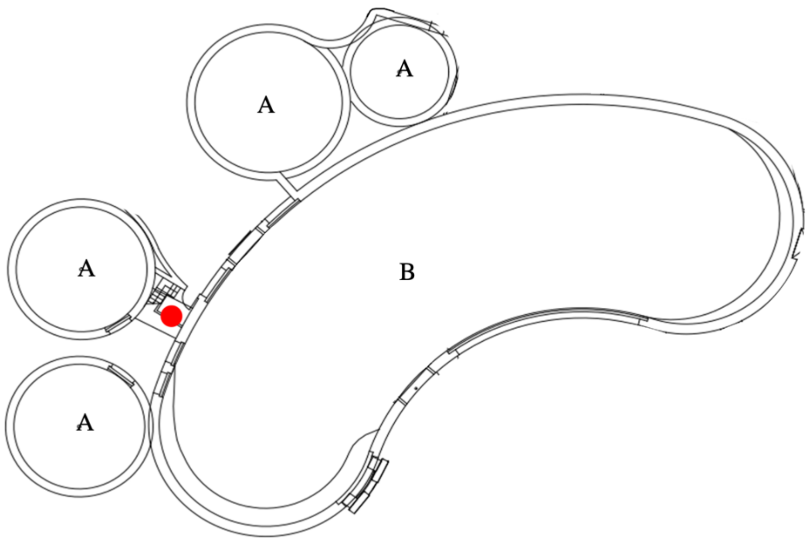

Figure. 3 The floor plan of the aquarium. A and B are the pools where beluga live in. Red spot was where SM4 and hydrophone placed.

2.8 Statistical analysis

F-test was used to assess the difference slope of standard solution and pooled scrape extracts in papalism. The sampling location variation (flipper vs. fluke) was analyzed by

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a linear regression. Descriptive statistics, analysis of variance and Kruskal–Wallis test were applied to cortisol samples. Chi-Square test of Homogeneity was used to analysis the variation among the acoustic activities in an event. Qualitative acoustic activities data were further analyzed using a principal components analysis (PCA).

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Section3. Result

3.1 EIA performance assessments

The estimated extraction efficiencies for skin samples (41.6%±15.9%) were based on recovering 320 pg of cortisol spiked 15 extracts. These measured extraction efficiencies were in turn used as a correction factor applied to all blubber and scrape cortisol measurements within this study. The EIA standards and the pooled serially scrape extracts exhibited statistical parallelism when analyzed via F-test. No significant differences ($p>0.05$, Figure. 4) was found in slope between the linear portions of the binding curves of serially diluted scrape extract as compared to pure hormone standards run un the same assay. No obvious trend was found in expected concentration when the standard solution was spiked with increasing amounts of blubber extract ($r^2 = 0.0537$ 118.8±11.2 pg/ml); a finding consistent with little or no evidence of matrix interference.

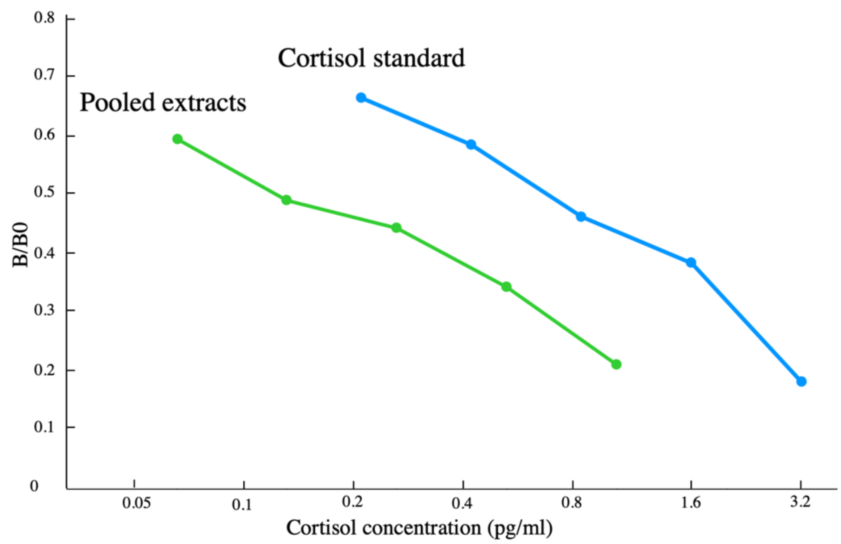

Figure. 4 Results from linearity assessment of EIA standard with scraped extracts.

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3.2 Scrape cortisol

The relationship between two sampling locations (flipper and fluke) was analyzed by linear regression. The regression showed the model fits the data very well (Figure. 5, R^2=0.9685). Thus, the cortisol concentration of the samples from these two locations could be similar.

The scrape samples (n = 92) from three captive beluga whales ranged from 0.213ng/g to 8.55ng/g (wet weight, wt). The cortisol concentrations did not differ between three beluga whales (Kruskal–Wallis test: H = 1.28, $p > 0.05$) (Figure. 6). If the value is below Q1 − 1.5 IQR or above Q3 + 1.5 IQR, it is viewed as an outlier, while IQR is interquartile range (Q3–Q1). In beluga A, there was one outlier and the CV was 67%. In beluga B and C, there were three and four outliers and the CV were 113% and 125%, respectively. When the outliers were not included, there was no differences in cortisol concentration between three belugas whales (Kruskal–Wallis test: H = 1.19, $p > 0.05$) and the CV of beluga A, B and C were 54%, 63% and 41%. The line graphs of the three beluga whales were presented in Figure. 7. The timeline of events was carefully compared with the line graph of cortisol concentrations (Figure. 7). It is noticed that 68-72 day was with the highest score (Table 1), indicating that the skin cortisol in beluga may present the stress response around 2 months ago. By comparing cortisol concentration result and

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husbandry record, nine events were identified.

Table. 1 Days of time lag and scores.

Day	Beluga whale			Score
	A	B	C	
28-32	2	3	2	7
38-42	2	5	2	9
48-52	2	3	3	8
58-62	3	4	3	10
68-72	3	6	5	14

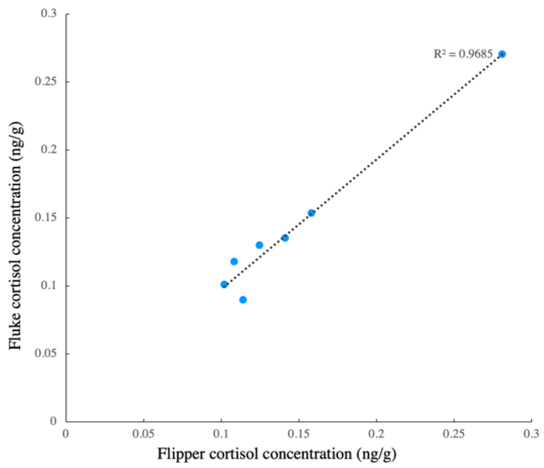

Figure. 5 Linear regression of flipper-fluke cortisol concentration.

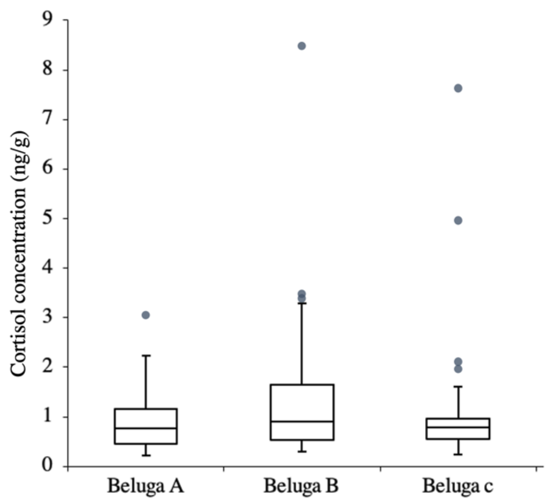

Figure. 6 Box plot of scrape cortisol concentration. Grey dots are the outliers.

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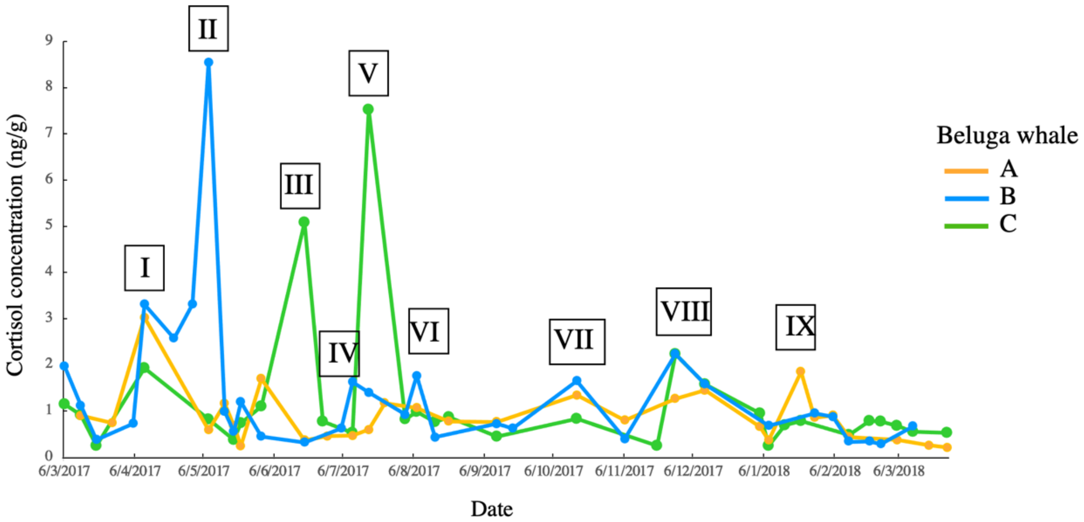

Figure. 7 Line graph of scrape cortisol concentration. Event I to IX were the identified event by matching peak of cortisol concentration and date of event .

3.3 Acoustic activities

Three events, which was filtering system maintenance infrastructure (event I), enteritis, which was diagnosed by veterinarian, of beluga C (event III) and unidentified noise from sea embankment (event IX) with high quality recording were selected for acoustic activities analysis. In total, 1430 calls were identified from the recoding of three events. Among these, pulse calls (1062), whistle call. (215), combo calls (141) and unknown (13) were identified. The significant difference in the total number of calls of five days was observed in event I, III and IX (Chi-Square test of Homogeneity: $p<0.05$) (Figure. 8). The 1st and 2nd axes of PCA separated acoustic activities into three clusters (Figure. 9). In these events, two days before the event were separated into two different

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clusters, and the day of events and two days after the event were separated into another cluster.

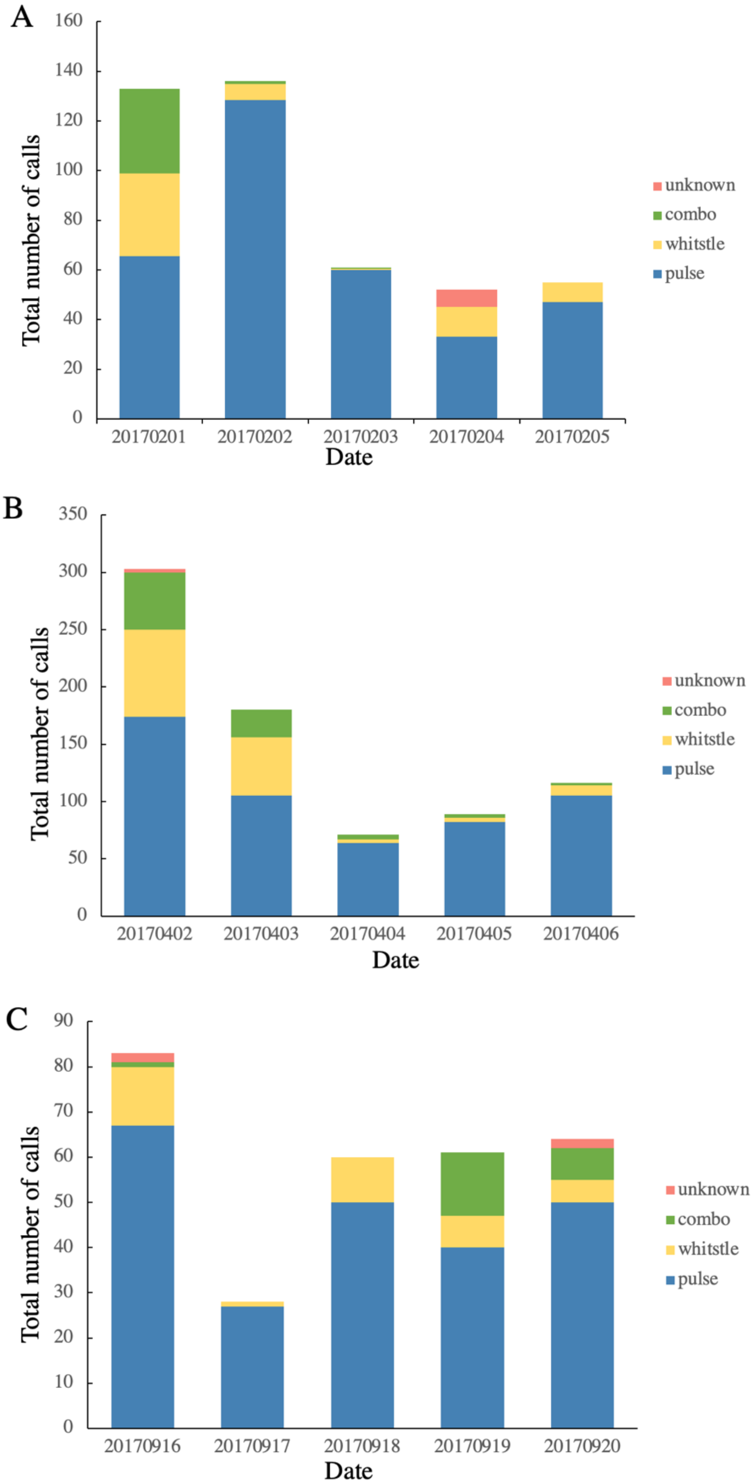

Figure. 8 The total number of calls and the pattern of acoustic activities in the analyzed events. A, B and C were event I, III and IX, respectively.

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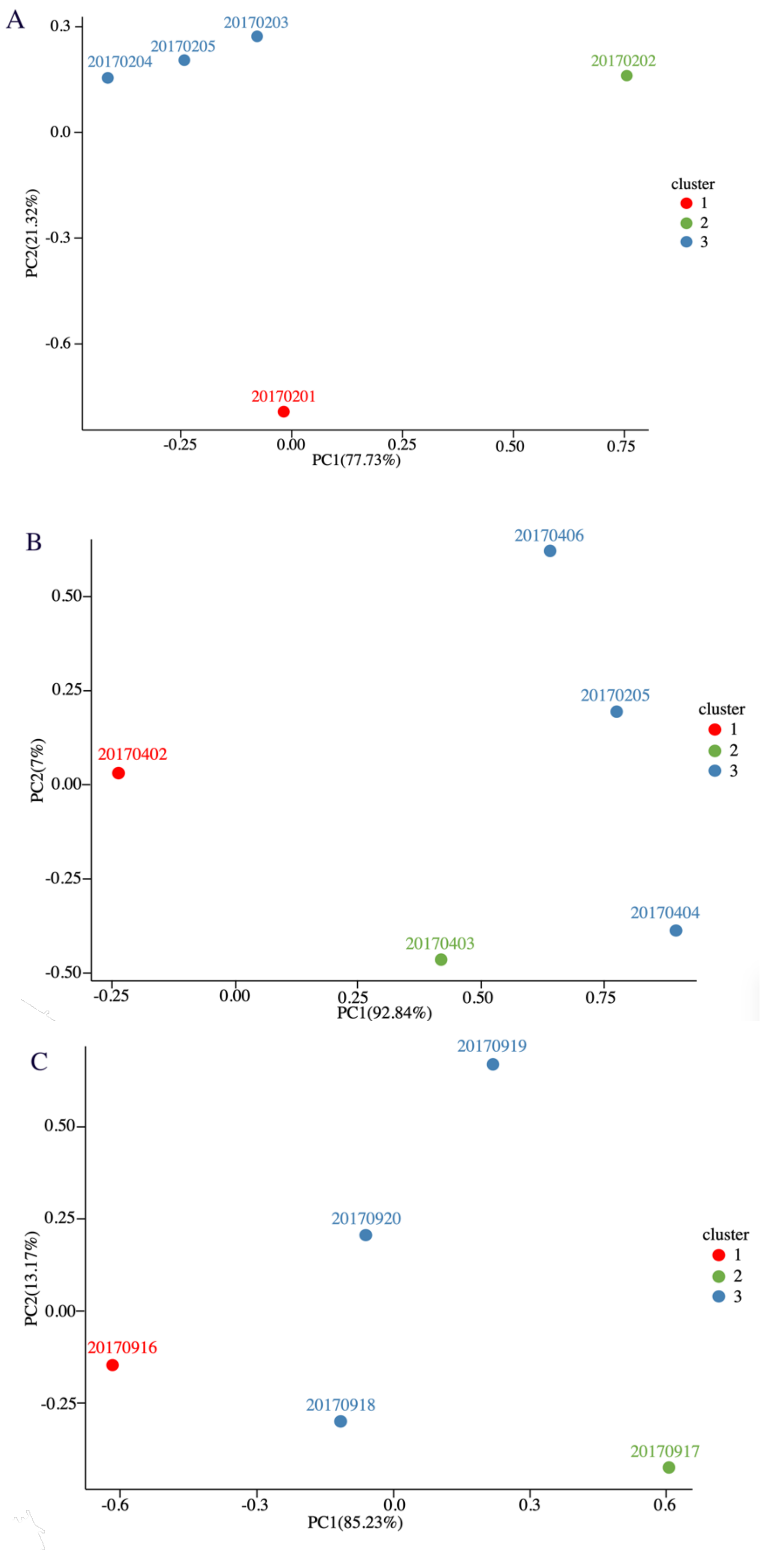

Figure. 9 Results of PCA analysis of different events. A, B and C were event I, III and IX, respectively.

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Section4. Discussion

The stress in captive cetaceans could not always be evaluated solely through clinical performance. Therefore, an effective method for measuring stress in captive cetaceans is important for maintaining good animal welfare. Simultaneously analyzing behavioral and physiological parameters has been considered as the most effective approach to measure welfare accurately (Castellote and Fossa, 2006). Approach with cortisol and specific behavioral monitoring could be helpful in representing the diverse and dynamic aspects of welfare experienced by the animal (Candiani *et al*., 2008). In terrestrial mammals, assessing stress and welfare by using physiological and behavioral parameters concurrently have been reported (Laws *et al*., 2007; Candiani *et al*., 2008; Haverbeke *et al*., 2008; Rajagopal *et al*., 2011; Salas *et al*., 2016). Meanwhile, there are only two published studies used multiple indicators in assessing captive cetaceans stress and welfare. One research investigated the correlation between stress and health by behavioral assessment and physiological data. The physiological measurement (blood tests and necropsy) were performed after the behavior changed (Waples and Gales, 2002). Another study demonstrate that the enclosure type (open and closed facilities) may influence the behavior and salivary cortisol concentrations of captive bottlenose dolphins (Ugaz *et al*., 2013). That result was suggested by comparing two indicators in group level. Here, we present the first study that used two indicators simultaneously to evaluate captive beluga

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whales stress and welfare in two levels, individual and group. The results presented here suggested that scrape cortisol measurement and acoustic recordings are a valuable method to monitor stress levels in beluga whales since the changes in the cortisol concentration and acoustic behavior could reflect the fluctuation of environment and body condition.

The three beluga whales had been in the same captive environment, so their stressors were assumed to be similar. However, their cortisol concentrations varied (Figure. 6). A possible explanation is the varied stress responses among individuals. According to the trainers, beluga B is more sensitive to environmental changes. The comparatively high cortisol concentration in some samples and high CV in beluga B indicates that this beluga likely showed a stronger response to certain events, while the other two beluga whales did not. Also, beluga C had some high cortisol concentration in some samples. However, the CV of beluga C drop 70% after the outliers were not included and beluga C had a lowest CV without outliers among three belugas. This showed that beluga C might only respond to significant stressors. Compared with the other two animals, beluga whale A only showed one outlier, suggesting this animal is relative stable as the trainers reported. Therefore, individual differences were also observed on the values of the CV of these three animals (Figure 6). This indicates that the animals' characteristics could be presented in the scrape cortisol concentration changes. It means that the scrape cortisol analysis could potentially be used in individual evaluation

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instead of group evaluation. In contrast, the current acoustic activities monitoring is a potential tool for group evaluation. It was beneficial that all acoustic activities from three beluga whales can be recorded by transducer. However, the limitation of measuring acoustic activities only would bring difficulties in evaluating each beluga stress response. Moreover, each animals' response to the changes of welfare and stress will be modified by a number of factors, and so the evaluation of the response will need to include consideration of individual details of the anima (Wolfensohn *et al*., 2018). Thus, this provides evidence that using these two indicators could provide a more comprehensive evaluation of each beluga whale stress and welfare.

Enteritis was reputed as a physiological disorder that might cause a progressive effect on the animal's health and affect the total number of calls slowly. On the day with the lowest number of calls (4th April), it was suggested it was the day that beluga C was seriously discomforted and the trainer and veterinarian diagnosed the animal with enteritis. This was also observed that the total number of calls before the diagnosis was separated into different clusters in the PCA (Figure. 9B). Although enteritis was only diagnosed in beluga C only, the behavior changes (decreased of total number of calls) might be happened in the same group of beluga whales. Thus, a gradually decrease of total number of calls was observed in event III (Figure. 8B). In contrast, a sudden decrease of the total number of calls was observed in event I (filtering system maintenance

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infrastructure) and event IX (unidentified noise from sea embankment). In event I, the total number of calls before the event was separated into two clusters (Figure. 9A) and the call pattern varied (Figure. 8A). A possible explanation would be the normal variation among different days. When matching the husbandry and medical record, the date of the event I, III and IX was suggested to be 3rd February, 4th April and 18th September, respectively. However, in event IX, the total number of call decrease on 17th instead of 18th September. Therefore, it is possible that the environmental noise might begin on 17th. Although the after-event acoustic activities (include the date of the event and after the event) was decreased in all types of calls and grouped into a cluster in PCA, event I was considered as the most serious event. The significantly low total number of calls in event I appeared for at least two days after the event happened, but the total number of calls and the variety of calls had recovered quicker in event III and event IX (Figure. 8). After event I happened, combo call did not recover after two days, meanwhile, combo and whistle call began to recover in event III and IX. This may indicate different event may had different effect on the changes of the call pattern and the event which took place within the aquarium might have a longer negative effect on the animals. A previous study investigated the construction effect in captive terrestrial mammals and suggested the changes of behavior after exposure to construction noise potentially indicative of agitation or stress (Jakob-Hoff *et al*., 2019). During the maintenance, the noise or

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vibration would stress the beluga whales. When the construction or maintenance is necessary, the aquarium should take precautions to minimize the duration and intensity of exposure. Further analysis of acoustic activities may build up the group acoustic base, including total number of calls and pattern of calls at different timing within a day, for understanding which recording within a day could best represent the day. In the current study, it is suggested that when the beluga was stressed, the total number of calls may decrease. Studying the changes of acoustic activities in different event would have a better understanding on the effect caused by the stressor. Also, investigating the time for acoustic activities return to normal range would help understanding the duration of the effect. This would help mitigate the stressors by grading different events and improve the management in the captive environment.

Understanding how long the time of cortisol signal appears in scrape sample after a stress event is important to identify the cause of stress. It is believed that the incorporation of cortisol (and other hormones) into the skin is due to skin renewal, which can deliver hormones to the margin of the skin (Bechshoft *et al*., 2020). Although the estimates of epidermal turnover rate ranged from 70-75 days (Hicks *et al*., 1985; Aubin *et al*., 1990), which was estimated by sub-epidermally injecting labeling substance in bottlenose dolphin and incorporating [6-^{3}H]thymidine into the nuclei of cells synthesizing DNA in beluga whales, the latest research suggested the time lag in bottlenose dolphin is

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around 45–60 days (Bechshoft *et al*., 2020). Meanwhile, In the current study, the time lag in beluga whales is suggested to be 68-72 days, which is similar to the time lag in a previous study (Aubin *et al*., 1990). The comparatively longer time lag in beluga may be similar with the reason of longer wound healing time, which is due to the thickness of the beluga epidermis and the intrinsic rate of growth of basal cell is slower (Geraci and Bruce-Allen, 1987). This known lag provides an opportunity to assess the animal response to a stress event long after other measurements (e.g. cortisol in blood and feces) have returned to baseline in a short period. According to the husbandry record, the trainers did not record any abnormal behavior in the three event which analyzed in the current study. Through routine scrape cortisol concentration monitoring and acoustic base, the stress response of beluga whales could have better understanding from an objective point of view, instead of relying on personal judgment only.

During the sample collection, collection from exactly the same location for each individual is often required. According to the pre-sampling training in scrape sample collection, after once collection, it took nearly one week for generating enough scrape for cortisol measurement. Collecting samples from the same location would increase the time interval for each sample. Also, the sample from a single location may be limited and not enough for the cortisol extraction. Thus, it would be difficult to maintain a routine sample for setting up the fine scale baseline for evaluating the stress. In the current study, no

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portion of variation was found between the sampling from flipper and fluke (Figure. 5). This showed that trainers would have flexibility to collect samples from either flippers or fluke to represent the day. Also, when there were limited samples in single location, samples from both locations (fluke and flipper) may be able to mixed and shorten the interval between each collection. Also, to the best of our knowledge, the only study about cortisol distribution in cetacean skin suggested no difference in blubber cortisol concentration across dorsal, lateral, and ventral around the girth of the animal(Kershaw *et al*., 2017). According to findings in this study and our study, it is hypothesized that there is no difference of cortisol concentration in the skin of whole body in cetaceans although it needs further research.

The pooled extracts for parallelism were from a stranded pygmy killer whale instead of captive beluga whales. This was because the cortisol concentration from beluga whales was too low after the serial dilution and out of the range of the EIA detection. The scrape cortisol concentration presented in current study 0.213ng/g to 8.55ng/g (wt) was similar to previous studies, which was 0.6-15ng/g (dry weight, dw) in bycatch harbor porpoise (Bechshoft *et al*., 2015) and 0-8.4ng/g (dw) in captive bottlenose dolphin (Bechshoft *et al*., 2020). Meanwhile, although comparatively low concentrations (medium value: 1.14 ng/g) were detected in scrape samples, the changes could be detected

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by EIA. This provides evidence that EIA could be used to monitor the dynamic changes of scrape cortisol concentration.

A previous study using a similar extraction protocol in homogenizing blubber samples and the extraction efficiency was 68.5% ±13.9% (Kellar *et al.*, 2015). Moreover, the lipid content is inversely proportional to the duration of exposure to seawater (Ryan *et al.*, 2013). In the current study, the relatively low extraction efficiency in scrape samples (41.6%±15.9%) might be due to the exposure to seawater. Therefore, after the collection of a scrape sample, centrifugation is necessary to remove seawater. Because the scrape sample is in liquid-solid form, seawater was observed in the upper layer after centrifugation. Therefore, after collecting scrape samples, the removal of seawater is not only necessary but should be done rapidly. Otherwise, the cortisol concentration would be underestimated due to lipid loss and the stress analysis results would be erroneous.

GCs are the principal glucocorticoids secreted by the adrenal gland, through activation of the HPA axis (Martin and Crump, 2003). Cortisone is the inactive form of cortisol and corticosterone (Stewart and Mason, 1995). However, which GCs predominates varies among different animal species; for example, cortisol is the major component in humans, dogs, and cats, while corticosterone is the major one in rabbits and rats(Pineda, 2003). Only a few research mentioned that cortisol, corticosterone, and aldosterone involved in the cetacean stress response while progesterone is not (Hunt *et*

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al., 2018; Bechshoft *et al*., 2020). Meanwhile, the correlation of GCs in baleen varied. Previous study showed no significant correlation of cortisol to corticosterone in bowhead, while NARW and blue whale did have significant correlations(Hunt *et al*., 2018). Research from terrestrial mammals indicated that GCs may respond quite differently to stressors(Koren *et al*., 2012). To the best of our knowledge, there is no research on the ratio of cortisol to corticosterone in cetacean skin and blubber. According to the instructions provided by the manufacturer, the EIA kit used here has 100% cross-reactivity to cortisol, 18.8% with dexamethasone, 7.8% with prednisolone, 1.2% with corticosterone and cortisone and <0.1% with progesterone. Therefore, it is unclear if a certain percentage of the signals are from corticosterone and cortisone in the current study. There is thus a need to investigate the predominant GCs in cetaceans, enabling us to achieve more precise measurement of stress hormone concentration in cetacean skin and to evaluate the stress response.

This study used two non-invasive methods, including measuring acoustic activities and scrape cortisol, to evaluate the stress caused by different events. The three analyzed events showed an increase in the scrape cortisol concentration and a decrease in acoustic activities. Cortisol being the most common stress indicator may suggest the animal was responding to an event, and the acoustic activities could provide evidence that an event happened and cause negative/no effect to the animal. It is recommended that aquaria may

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consider incorporating these indicators to monitor stress and animal welfare. With well-trained personnel and proper equipment for efficiently collecting data and analyzing data, this method would substantially contribute to cetacean husbandry. This study may be the fundamental information of future studies on stress and welfare science for captive cetaceans, which can be used to monitor and subsequently improve their quality of life.

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