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Social, Reproductive and Contextual Influences on Fecal Glucocorticoid Metabolites in Captive Yangtze Finless Porpoises (*Neophocaena asiaeorientalis asiaeorientalis*) and Bottlenose Dolphins (*Tursiops truncatus*)

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Abstract: Although the use of fecal glucocorticoid metabolite (FGCM) measurements as non-invasive biomarkers for the stress response in mammals has increased, few studies have been conducted in odontocetes. We investigated if animal sex, age, pregnancy or contextual variations (season, sampling time, enrichment, social separation and presence of visitors) influenced the FGCM concentrations in presumably healthy, captive and endangered Yangtze finless porpoises (YFPs, N = 4) and bottlenose dolphins (BDs, N = 3). For YFPs, the FGCM concentrations were influenced by season ($p = 0.01$), diurnal variation ($p = 0.01$) and pregnancy ($p = 0.005$). Contextual variables that were associated with increases in FGCM concentrations included social separations ($p = 0.003$) and numbers of visitors ($p = 0.0002$). Concentrations of FGCMs were lower ($p = 0.001$) after exposure to environmental enrichment. For BDs, enrichment was associated with reduced concentrations of FGCMs ($p < 0.0001$). The presence of visitors also influenced this species' FGCM concentrations ($p = 0.006$). These results demonstrate that changes in the FGCM concentrations in YFPs and BDs may occur in response to contextual and social changes. In combination with other behavioral and physiological assessments, measurements of FGCMs may be a useful tool for monitoring cetacean welfare. Such monitoring may help researchers identify and better understand situations that may be stressful for animals and, therefore, improve management and husbandry. Furthermore, results from our study and inferences of the FGCM concentrations in cetaceans, and their potential relationship to stress, may be extrapolated to studies of free-ranging animals, which may help detect possible environmental or anthropogenic stressors that could be affecting these populations.

Keywords: bottlenose dolphin; fecal glucocorticoid metabolites; finless porpoise; enrichment; separation; welfare

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1. Introduction

In mammals, physiological or psychological stress is defined as unpredictable and/or uncontrollable stimuli [1] that activates the hypothalamic–pituitary–adrenal axis (HPA). Such stress results in the release of multiple hormones designed to help regulate physiological allostasis, principally through increasing metabolic activity and energy availability [2–4]. Once the HPA system is activated,

adrenocorticotropic hormone stimulates the release of glucocorticoids (GCs), and to a lesser extent in some cetaceans, including the bottlenose dolphin (BD, *Tursiops truncatus*) and the killer whale (*Orcinus orca*), the mineralocorticoid aldosterone ([5,6] for BD; [7] for killer whale). The allostatic response, characterized by releases of GCs to an acute stimulus, is adaptive, rapid and short in duration (minutes to hours). Conversely, elevated GC concentrations in response to repeated, chronic or long-term stimulation (several hours per day for weeks, months or longer) has been described as homeostatic overload [3,8]. This homeostatic overload can negatively impact multiple physiological systems (e.g., immunological, reproductive, circulatory), animal growth and cognitive and/or behavioral functions [9–14].

Increased GC concentrations can be detected within 5 min of HPA activation [15], last longer in duration (hours) and quantified either directly in serum or indirectly via excreted metabolic products [16]. Because GCs are generally accepted as an indirect, measurable index of an organism's allostatic response toward positive (e.g., feeding) or negative stimuli (e.g., stressful events), changes in the concentrations of these hormones may provide valuable information regarding the impact of anthropogenic and environmental disturbances on individuals. However, because both positive and negative stimuli can trigger this allostatic feedback system, contextual and behavioral information must be combined with hormonal changes and other physiological indices of health before these fluctuations are used to analyze and assess their welfare [17–20]. In cetaceans, studies of GCs, or their related excreted metabolites, have been used to characterize: diurnal variation, stress-induced changes and the influence of transport on serum or exhaled respiratory vapor GC concentrations in belugas (*Delphinapterus leucas*, [21–24]); diurnal and seasonal influenced changes on circulating GCs [25,26], pregnancy variations and impacts of inadequate prey and boats in killer whales [27–29]; sex, age, season, cold stress, facility type, administration of bovine lactoferrin or capture effects in BDs [5,25,30–35]; and seasonal variations and capture effects in Yangtze finless porpoises (*Neophocaena asiaeorientalis asiaeorientalis*, YFPs) [36]. However, the study of GC concentration fluctuations, depending on the parameters evaluated, has not always revealed the same patterns (e.g., pregnancy in BDs [29]) and, therefore, more studies are needed to understand these variations.

Although circulating GC concentrations can be an indicator of the HPA response toward a stressor, in wildlife species the actual collection process, which usually involves capture and restraint, may cause a stress response itself, thereby masking the animals' normal concentrations [37,38]. This limitation has led to the use of less invasive methods for determining an animal's allostatic state by relying on fecal GC metabolic products in multiple terrestrial species (see review of FGCM studies [39]) and a few cetacean species [6,7,34,40,41]. FGCM concentrations are influenced by pregnancy and/or parturition in BDs [41], humpback whales (*Megaptera novaeangliae*) [42,43] and blue whales (*Balaenoptera musculus*) [44]. A study in the North Atlantic right whale (*Eubalaena glacialis*) has demonstrated that FGCM concentrations are influenced by loud vessel noise or by animal entanglement in fishing gear [40,42,44]. Finally, in killer whales, a purported lack of adequate prey is believed to be primarily responsible for an increase in FGCM concentrations [28].

Captive cetaceans may face a variety of anthropogenic (e.g., animal transport, social isolation, lack of environmental enrichment, holding pool size and noise) and natural stimuli (e.g., seasonal environmental changes and conspecific interactions) that may or may not influence the HPA axis, depending on species and individual differences [24,32,45,46]. To better understand the factors that may be reflected in allostatic adjustments in odontocetes kept under human care and to improve the management strategies for these animals, the influence of potential stressors on GC concentrations should be investigated [32,47,48]. However, using GC concentration measurements to identify potential stressful events can only be accurate when the GC concentration changes have been overlaid against or controlled for natural, diurnal or seasonal changes and positive stimuli [49,50]. Additionally, although it is clear that short-term stressors at irregular intervals may be important for the overall fitness of both captive and wild animals, fitness deteriorates during chronic exposure toward any one or multiple stressor(s) [18,51,52]. Therefore, it is crucial to identify the physiological responses to situations and/or

experiences that may or may not elicit or be associated with an allostatic response. Once categorized, exposure to negative stimuli or stressors can be reduced or monitored in order to avoid chronicity and presumptively improve welfare.

Because different species, different groups and even different individuals may exhibit different stress responses, investigating these responses in every captive group is required, and broad generalizations of these stress responses might not be possible. This type of examination may be of importance for endangered species, like the YFP, whose ability to reproduce in captivity may be instrumental in preventing their extinction. Understanding how these different species and individual animals react to different environmental, physiological or husbandry events may aid in the development of more effective management programs that create an atmosphere best suited for successful reproduction, fecundity and overall welfare. Therefore, in this study, our objective was to investigate several contextual conditions that can occur during management of captive populations and their potential influence on FGCM concentrations in two species, the critically endangered YFP [53] and the BD.

2. Material and Methods

2.1. Subjects, Housing and Group Composition

Ethical approval (AW01-1819) for the study was obtained from the Research Ethic Committee of the Institute of Hydrobiology, University of the Chinese Academy of Sciences, Wuhan, China. The study strictly adhered to the Chinese Law and ethical guidelines for biodiversity. Fecal samples were collected from four YFPs living in the Baiji Dolphinarium, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China (Table 1), from September 2017 through October 2018. The group included five individuals, but one of the females was excluded from the study due to insufficient training for routine fecal sample collection. Due to group management reasons (i.e., management of pregnant females), the makeup of the social group changed several times during the study. The YFPs' facility's filtration and water treatment system consists of pressure sand filters and chlorine- and ozone-sterilization equipment. Water is filtered at least four times a day and varies between 12 °C in the Winter to 27 °C in the Summer. When all the individuals were housed together, the YFPs were kept in a kidney-shaped pool with a length (L) of 20 m, width (W) of 7 m and depth (D) of 3.5 m, linked by a corridor to a circular pool with a 10 m diameter and 3.5 m D. These two pools were separated by a gate that permitted visual and acoustic access by animals between the pools. A third, unconnected, circular pool (13 m diameter and 3.2 m D) was used from February 2017 to house the excluded female and male, MP2, until the female gave birth. Following the birth, the male was moved back to the other pools and the female and calf remained in the unconnected pool. Fecal samples were also collected from three male BDs living in Haichang Polar Ocean World, Wuhan, China (Table 1), from December 2017 through October 2018. The group included the three males and one female at the start of the study. A second female from another facility was added to the group at the end of January 2018. The BDs' facility's water was reconstituted sea water and was filtered around four times a day. Its temperature was maintained at 14 °C throughout the year. The BDs were kept in a three-pool complex, with two 8.86 m diameter, 5 m D, circular pools ("small pools") connected to the main pool that was 27.44 m L, 12 m W and 6 m D ("large pool"). Depending on the day and time, the animals had access to one, two or all pools. Upon arrival of the second female, the females were separated from the males and kept in one of the small pools while the males were in the other round pool and/or in the main pool. On two occasions, the social grouping changed for a few days (male MD2 with one female and males MD1 and MD3 with the other female).

Table 1. Catalogue of the individuals' features. N = number of fecal samples collected.

Name	Species	Sex	Age (Year)	Weight (kg)	Length (m)	N
MP1	Yangtze finless porpoise [a]	M	8	NA	1.57	44
FP1 *	Yangtze finless porpoise [a]	F	8	NA	1.45	70
MP2	Yangtze finless porpoise [a]	M	14	NA	1.56	24
FP2 *	Yangtze finless porpoise [a]	F	11	NA	1.47	7
MD1	Bottlenose dolphin [b]	M	13	280	2.74	4
MD2	Bottlenose dolphin [b]	M	14	290	2.69	35
MD3	Bottlenose dolphin [b]	M	13	260	2.70	10

* Pregnant females; [a] Baiji dolphinarium, Institute of Hydrobiology, Wuhan, China; [b] Haichang Wuhan Polar Ocean park, Wuhan, China.

For the YFPs, the animals participated in four to six training sessions a day with no public presentation, but occasionally visitors were allowed to watch animals from both the surface and underwater windows. Animals were fed between 3 and 3.5 kg of thawed and/or live fish (species: *Basilewsky*) per day during training sessions. For BDs, animals participated in three training sessions and between two and five public presentations per day. Visitor access to the BDs was only permitted approximately 20 min prior to a presentation and during presentations. They were fed between 10 and 13 kg of thawed fish (capelin, herring, squid and mackerel) per day during these training and presentation sessions.

During the first training session of the day, all animals were subjected to a routine body/health check, and the respiratory rate of each individual was recorded immediately following this session to assess the animals' health status. All the animals included in the study were presumed to be healthy based on the daily caretakers' checks and observations and veterinary examinations. Animal enrichment was provided in the form of human-made objects (e.g., toys) or live fish (for YFPs) at times decided by the caretakers who followed no schedule but opportunistically provided enrichment. Caretakers also frequently interacted with the BDs and YFPs outside of training sessions. All pools were frequently cleaned by divers and/or caretakers by scrubbing the upper part of the pools' walls, once a day for BDs and approximately once a month for YFPs.

2.2. Data Collection

2.2.1. Fecal Sample Collection

Sample collection was conducted over 14 months for YFPs and 10 months for BDs. Fecal sample collection was attempted daily for YFPs, although sample collection was not always successful and multiple collections throughout the day could rarely be accomplished due to the caretakers' responsibility for training other behaviors, lack of feces at the sampling time (the animal defecated before the training session) and cooperation from the animals. Collection attempts usually occurred in the early afternoon, but some samples were also collected in the morning and in the late afternoon (Table 2). Animals were trained to lay in a belly-up position and to freely defecate after a gentle

finger press next to their anus. Feces were then collected carefully with a plastic spoon to avoid water contamination. For BDs, fecal collection occurred twice a week, at approximately the same time (13:30–14:00). Animals were trained to lay in a belly-up position and a 3-mm internal diameter plastic tube was inserted into their anus to extract feces. Feces were not always able to be collected during every attempt. All samples were then stored at −20 °C within two to ten min of collection. A total of 145 samples were collected for YFPs and 49 for bottlenose dolphins (Table 1).

2.2.2. Contextual Events and Situations Associated with Fecal Samples

Based on previous research in BDs, which has demonstrated that the acute stress event's influence on the FGCM concentrations occur within ~4.5 h of a stressor being perceived [6], we associated the fecal samples with the contextual events that occurred between 4 h and 8 h before sample collection. For YFPs, samples collected in the early or late afternoon were associated with the morning context of that day (within 4 to 8 h of the morning time frame). For samples collected in the morning, samples were associated with the contextual events of the afternoon of the day before (approximately 12–15 h before sample collection). For BDs, all samples were collected in the early afternoon; therefore, each sample was always associated within the time frame of the morning of that day. The context was recorded following a series of questions related to social and environmental events or cues (Table 2). Enrichment was defined as the presence of "toys" (e.g., balls, buoys, etc.).

2.2.3. Sample Processing and GCMs Assay

To measure the FGCM concentrations in fecal samples, we followed the method from Ayres et al. [28] and Steinman et al. [7], with minor modifications. Samples were thawed, and the wet feces thoroughly mixed and homogenized. Approximately 0.5 g (finless porpoise range, 0.461–0.578 g; bottlenose dolphin range, 0.101–0.551 g) of wet feces was analyzed. For the BD fecal samples, the mean and median sample weights were 0.471 g and 0.512 g, respectively, and 4/49 samples (8.2% of total) had sample weights that were <0.25 g. For specific details of the extraction process, please refer to the aforementioned publications.

Extraction efficiency was measured. For the YFPs, four samples of wet feces (two from males and two from females) were used. Each sample was weighed (~0.5 g, 4 replicates per sample) and extracted as described above. A second set of samples (also ~0.5 g, 4 replicates per sample) was spiked with 30 ng of cortisol standard (Sigma Aldrich, St. Louis, MO, USA) and processed identically to the first set. There was a total of eight replicates for each sample. The mean ± SEM recovery of the cortisol for the spiked YFP fecal samples for males was 99.9 ± 10.1% and 110.9 ± 6.3% for females. For the BDs, a pool of wet feces from all the study males was made. Twelve samples (six normal and six spiked with 30 ng of cortisol standard) were extracted as described above. The mean ± SEM recovery of cortisol for the bottlenose dolphin samples was 99.6 ± 2.8%.

Table 2. The environmental and social factors' features for Yangtze finless porpoises (YFP) and bottlenose dolphins (BD)

Species	Sampling Time	Season	Social Grouping	Enrichment	Visitors
YFP	Morning (8:00–11:00)	Winter (December–February)	All together	None	None
	Early afternoon (14:00–15:30)	Spring (March–May)	Separated [a]	Toys [c]	Low [d]
	Late afternoon (16:00–17:30)	Summer (June–August)	Alone [b]		High [d]
		Fall (September–November)			
BD	Early afternoon (13:30–14:00)	Winter (December–February)	All together	None	Scale from 0 to 3 based on the public present during shows (0: park closed, "None", 1: one to two rows full of visitors, "Few", 2: three to six rows, "Moderate", 3: more than six rows, "Many")
		Spring (March–May)	Separated [a]	Toys [c]	
		Summer (June–August)			
		Fall (September–November)			

[a] Gate between groups allowing visual and acoustic contact.; [b] Single animal with gate allowing visual and acoustic contact to other animals.; [c] For YFP, balls. For BD, balls, buoys and ropes + buoys.; [d] Low < 5 people in front of underwater windows or next to pool; High > 5 people.

The GCM concentrations in the feces were measured using an in-house, single antibody, direct enzyme immunoassay (EIA, [54]) previously used for killer whale feces and described in Steinman et al. [7]. For females, sample dilutions containing 0.0005 to 0.002 mL of fecal extracts or 0.002 mL for males were analyzed. Parallel displacement of the extracted feces compared to the standard curve was demonstrated (r = 0.999 (YFP and BD males) and 0.995 (females), $p < 0.05$), and an accuracy test plotting the concentrations of finless porpoise fecal extracts spiked with known concentrations of the standard against the non-spiked fecal extracts yielded a recovery of 92.17 ± 8.53% (linear regression, $y = 0.96x − 3.89$, $r^2 = 0.998$) for YFP males, 97.62 ± 10.82% (linear regression, $y = 0.94x + 4.23$, $r^2 = 0.999$) for BD and 107.96 ± 4.85% (linear regression, $y = 1.16x − 8.37$, $r^2 = 0.991$) for YFP females. Assay sensitivity was 3.9 pg/well. To test for intra-assay variation, two pools of fecal extracts (one female, one male) were run across various, randomized wells (N = 16 for each sex) on the microtiter plate. The mean concentration for each sex was determined from all replicates and the coefficient of variation (CV) was calculated by dividing the mean by the standard deviation. The CV was expressed as a percentage and was 4.7% for females and 5.1% for males. Inter-assay CVs for the two quality controls binding at 30% and 70% were 6.3% and 12.2% (N = 11). Species-specific biological controls were made up of the finless porpoise fecal extracts and, to test for both species and matrix-specific variation, had inter-assay CVs of 9.2 and 12.6% (N = 6, 5) for males and females, respectively. Hormone results are expressed as ng FGCM/g wet, extracted feces.

2.3. Statistical Analyses

Data were analyzed using Stata®(version 16; StataCorp LP, College Station, TX, USA). For detection of any significant associations between the FGCM concentrations and contextual variables we used a two-stage restricted maximum likelihood (REML) linear mixed effects (LMM) regression model [55], with animal ID as the random intercept. Linear mixed models where slopes varied significantly by individual, as determined by the likelihood ratio analysis, were run using both random slopes and random intercepts. The fixed effects portion of the model included the dependent variable FGCM concentrations and the independent variables animal age (continuous variable), sex (YFP only, coded 0, 1), season (coded 0 to 3), diurnal (YFP only, coded 0, 1, 2), unusual events (Y or N, coded 0, 1), unusual event type (coded 0 to 5), separation (coded 0, 1, 2), public (YFP only, coded 0, 1, 2), pool size (BD only, coded 0, 1, 2) and toys (Y or N, coded 0, 1; Table 2).

Test for global significance of each categorical variable was done post hoc using Wald χ^2 test [56]. Non-significant variables with a probability value of greater than 20% were iteratively removed (reduced model) in a stepwise backward progression. The effect of removing these variables on the model with all fixed variables (full) was evaluated using the LR test [55] and the lowest Akaike's information criterion [57]. The variable was retained if removing it had significant effects on the model.

For physiologic validation, concentrations of FGCM during pregnancy, in non-pregnant females and males were compared using REML LMM with animal ID as the random intercept (and/or slope) variable. The fixed portion of the model included the dependent variable FGCM concentration and the independent categorical variables season and status (male, female, pregnant female and male bottlenose dolphin, coded 0 to 4). The FGCM concentrations from the male bottlenose dolphin were added for inter-species comparison.

In order to evaluate the FGCM concentration changes during pregnancy in the YFP, samples were indexed based on day prior to parturition (PP) and then partitioned into month prior (MPP, month 0 being the month of parturition) and 3 equal periods (stages) based on an estimated 11-month gestation [58]. Samples were binned into trimester as follows: early stage, 330 to 221 days PP; mid stage, 220 to 111 days PP; and late stage, 110 days PP. In addition to samples collected during pregnancy, samples collected during non-pregnancy were grouped together and included in the categorical variables as either MPP 12 or Stage 4 as appropriate. For the analysis of FGCM concentration changes during either trimester or MPP, a REML LMM was used as described above and included the independent fixed categorical variables season and either MPP or trimester.

After all the model development, quantile–quantile (qnorm) plots of the standardized residuals were evaluated for normal distribution; if non-normal, the data were transformed as determined by the Shapiro–Wilk test until the residuals were normalized, and the final model was then re-analyzed. Finally, pairwise comparisons of the marginal means between and within the fixed variables were made while applying the Šidák correction factor. Unless specified, the data in the tables and figures are presented as the marginal mean ± SEM and 95% confidence intervals (CI), and significance was set at $p \leq 0.05$.

3. Results

Concentrations of FGCM ranged from 42 to 720 ng/g for YFPs and from 25 to 338 ng/g for BDs. The final linear mixed model used for the YFP analysis was significant (Wald $\chi^2 = 467$, $p < 0.0001$) and included a second stage consisting of animal ID as the random intercept; covariance was set as unstructured, and residuals were set as independent by time of day. The residuals of the final model were normalized using log FGCM transformation. For the BDs, the final model was significant (Wald $\chi^2 = 60$, $p < 0.0001$), with animal ID as the random intercept, covariance unstructured and residuals set as independent by ID. The residuals of the final model were normalized using log FGCM transformation.

3.1. FGCM Concentration Characteristics and Physiological Validations

Age did not influence the FGCM concentrations for YFPs ($\chi^2 = 1.5$, $p = 0.22$) or BDs ($\chi^2 = 0.35$, $p = 0.55$). Status influenced ($\chi^2 = 55.6$, $p < 0.0001$) the FGCM concentrations in the YFP and BD, whereby pregnant YFPs had significantly greater concentrations than non-pregnant YFP females, YFP males and BD males ($p < 0.005$). No other intra-status significant differences were detected (Table 3, Figure 1A).

Table 3. Marginal means and 95% confidence intervals (CI) of fecal glucocorticoid metabolite (FGCM) concentrations (ng/g) for each stage of pregnancy and month prior to parturition (MPP) for Yangtze finless porpoise. N = number of fecal samples collected. Superscripts (A,B) indicate statistically significant differences (Šidák groups, $p < 0.05$) within each category. Data were first log-transformed for analysis, then back-transformed.

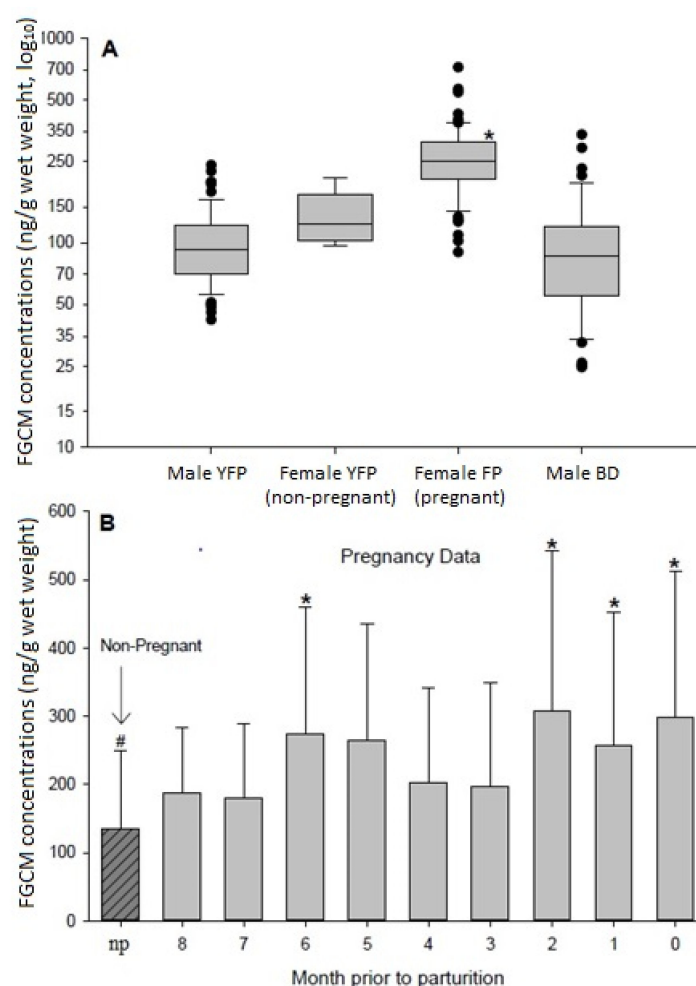| Category	Condition	N	Marginal Mean FGCM	95% CI
Stage of Pregnancy	Non-pregnant	8	131 [A]	107–161
	Early	14	202 [A, B]	127–321
	Mid	29	243 [B]	206–286
	Late	24	253 [B]	211–302
MPP	Non-pregnant	8	135 [A]	114–161
	8	2	188 [A, B]	95–372
	7	4	180 [A, B]	109–298
	6	8	273 [B]	186–403
	5	5	264 [A, B]	172–406
	4	9	202 [A, B]	139–293
	3	15	197 [A, B]	152–255
	2	11	308 [B]	234–403
	1	9	256 [B]	195–341
	0	4	299 [B]	213–341

Figure 1. (**A**) Box plot of the log-transformed fecal glucocorticoid metabolite (FGCM) concentrations for Yangtze finless porpoise (YFP) and bottlenose dolphin (BD). For box plots, the line inside the box denotes the median value, the height of the box represents the 25th and 75th quartiles and whiskers represent extreme observations (10th and 90th percentiles). Closed circles represent outliers. Asterisk (*) represents a category that is statistically different (Šidák groups, $p < 0.05$,). (**B**) Mean ± SEM FGCM concentrations during YFP pregnancy. Months prior to parturition with asterisks (*) are statistically different ($p < 0.05$) compared to the non-pregnant concentrations (#). Data were log-transformed for analysis, then back-transformed.

Both stage of pregnancy ($\chi^2 = 32.1$, $p < 0.0001$) and month prior to parturition (MPP, $\chi^2 = 45$, $p < 0.0001$) influenced the FGCM concentrations compared to non-pregnancy. For stage of pregnancy, the FGCM concentrations increased during each successive stage, with concentrations being significantly higher during mid and late stage ($p < 0.001$) compared to non-pregnancy (Table 3). Within MPP, significant increases in FGCM concentrations, as compared to non-pregnancy, occurred during MPPs 6, 2, 1 and 0 ($p < 0.015$, Figure 1B). The peak concentration occurred in MPPs 2 and 0 (Table 3); however, no samples were collected within 25 days of parturition.

3.2. FGCM Concentrations and Contextual Data

Season influenced the FGCM concentrations ($\chi^2 = 11.5$, $p = 0.01$) in the YFP, and the FGCM was higher ($p < 0.05$) in winter and spring compared to fall (Table 4). Season had no influence on BDs' FGCM concentrations. For the YFPs, there was an association of sampling time ($\chi^2 = 8.6$, $p = 0.01$, Table 4, Figure 1), where the FGCM concentrations were higher in the morning ($p < 0.05$) compared to mid-day. There was an association with enrichment, whereby the FGCM concentrations were reduced

for YFPs (χ^2 = 5.0, p = 0.001) and BDs (χ^2 = 28.6, p < 0.0001) (Table 4). There was an association of animal separation (χ^2 = 11.9, p = 0.003) and FGCM concentrations in YFPs, whereby the FGCM concentrations were higher when the animals were separated compared to when the animals were all together (Table 4). Separation had no effect on the FGCM concentrations in BDs. There was an association of the number of visitors and FGCM concentrations for both YFPs (χ^2 = 17.5, p = 0.0002) and BDs (χ^2 = 12.4, p = 0.006, Table 3). For the YFPs, the FGCM concentrations exhibited a U-shaped curve with concentrations reduced when few visitors were present compared to no or many visitors (p < 0.05). For BDs, the lowest FGCM concentrations were associated with times when no visitors were present and when the show audience capacity was between three to six rows, followed by an audience capacity of more than six full rows and an audience capacity of one to two rows (p < 0.05).

Table 4. Marginal means and 95% confidence intervals of the FGCM concentrations (ng/g) for each status, environmental and social condition for Yangtze finless porpoise (YFP) and bottlenose dolphin (BD). N = number of fecal samples collected. Superscripts (^{A,B,C}) indicates statistical significant differences (Šidák groups, p < 0.05) within each category.

Category	Species	Condition	N	Marginal Mean	95% CI
Status	YFP & BD	Pregnant Female	67	236 ^{A}	190–293
		Non-pregnant Female	8	134 ^{B}	93–194
		YFP Male	69	100 ^{B}	83–121
		BD Male	49	89 ^{B}	73–109
Season	YFP	Winter	55	165 ^{A}	154–175
		Spring	29	143 ^{A,B}	125–164
		Summer	5	111 ^{A,B}	83–147
		Fall	56	143^B	126–162
Time of day	YFP	Morning	15	176 ^{A}	149–207
		Early afternoon	94	141 ^{B}	132–151
		Late afternoon	36	152 $^{A, B}$	137–170
Enrichment	YFP	Absent	106	155 ^{A}	146–165
		Present	39	136 ^{B}	123–150
		All together	86	140 ^{A}	130–150
Social grouping	YFP	Separated	33	153 ^{B}	137–171
		Alone	26	182 ^{B}	159–209
Vistors	YFP	None	72	151 ^{A}	140–162
		Few	30	122 ^{B}	109–138
		Many	43	170 ^{C}	154–188
Season	BD	Winter	20	99	82–120
		Spring	12	82	62–109
		Summer	11	72	54–95
Enrichment	BD	Absent	20	135 ^{A}	109–166
		Present	30	61 ^{B}	52–73
Visitors	BD	None	15	70 ^{A}	56–87
		Few	14	113 ^{B}	90–142
		Moderate	5	64 ^{A}	44–92
		Many	11	99 ^{A,B}	75–130

4. Discussion

This study presents the results of the first analysis of FGCM concentration measurements in YFPs. We found an influence of pregnancy on FGCM concentrations, where pregnant females had the highest concentrations of FGCMs. In addition, various contextual, social and environmental conditions that these captive populations experienced and their association with the FGCM concentrations were investigated, revealing significant links between the FGCM concentrations and context.

In a previous study in the BD, using a radioimmunoassay (RIA), the FGCM concentrations ranged between 150 to 4450 ng/g with a maximum concentration of 21,000 ng/g after a stress test [6]. A range of 500 to 5000 ng/g for RIA-assayed FGCM concentrations in wild killer whales has been reported [28]. In zoo-based killer whales, and using similar sample processing methods and the same assay methodology in the present study, ranges of 38 to 571 ng/g for the FGCM concentrations during a biological stress response test have been reported [7], similar to the ranges reported here for both the YFP and BD. However, in another study, the RIA-assayed FGCMs ranged between 0.2 to 9.5 ng/g for five, healthy BDs [41]. The different ranges given by Champagne et al. [6] and Biancani et al. [41] might be explained by the differences between facilities or the features of the studied animals (e.g., sex, age and dominance status). However, the methods used to process and extract cortisol and its metabolites from feces and the analyses (e.g., EIA versus RIA, varying antibody cross reactivity) can lead to differing quantitative results. As a result, direct quantitative comparisons of the FGCM concentrations between studies may not be appropriate, but when a large enough data set is available, comparisons of trends and profiles are possible. Moreover, other factors that we did not include in our analysis may have also played a role and influenced the FGCM concentrations (e.g., type of food), making any comparisons irrelevant.

4.1. FGCM Concentrations Biological Validation

Due to the fractious nature of the YFP and the difficulties in behaviorally collecting multiple fecal samples during a 24-h period, a definitive biologic validation in the form of a stress test or hormone challenge similar to what has been done with BDs [6,59] and killer whales [7] could not be performed in these critically endangered animals. Paired blood collection, to use as a comparison between circulating GCs and excreted metabolites, was also not possible. However, we were able to provide a physiologic validation of our FGCM assay system by detecting significantly increased hormone concentrations during pregnancy as compared to non-pregnant females and males. This agrees with circulating GC and excreted GCM changes that have been documented during pregnancy in other cetaceans [29,41,43,60,61]. In addition, and as would be expected, we saw increased concentrations during the mid and late pregnancy stages compared to the early stage, and concentrations significantly increased during the final months prior to parturition (Table 3, Figure 1B). However, we were not able to collect samples within the last 25 days prior to parturition, so we were unable to determine if there was a surge in FGCM measures similar to what has been observed for circulating GC concentrations just prior to the onset of labor in other cetaceans and some domestic species [6,7,62,63]. Despite this lack of data during the final month, the increase in FGCM concentrations observed as gestation progressed, a phenomenon that is common during pregnancy in many mammalian species, is believed to be critical for final fetal respiratory system development and maturation, as well as maternal preparation for parturition [64–66]. Biological validation for BDs was not possible in our study but has been performed previously for this species [6,59].

4.2. FGCM Concentrations and Contextual Data

Because the animals were approximately the same age in each group (no young or old animals), the absence of an influence of age on FGCM concentrations was expected. Previous studies in BDs have also found no influence of age on FGCM or circulating GC concentrations [41,67]. However, future research with a larger number of animals and across a broader spectrum of age classes may reveal discrete influences of age, especially with respect to aged animals, as has been shown with circulating GCs in male killer whales [68].

The literature is mixed regarding seasonal influences on GCs in the BD. Similar to what we found for YFPs, the GCM concentrations were higher during the winter in some studies [35,69,70]. However, another study has found no clear seasonal pattern of GC concentrations in the BD, similar to our results [30]. Our findings of no difference in YFP FGCM concentrations between spring and fall is further corroborated by a study of wild male YFPs, which found no difference in the circulating

GC concentrations from animals sampled only during the spring and fall [36]. The lack of seasonal influences observed with the BDs may be due to lack of environmental changes. Animals had consistent light schedules (i.e., artificial light) and water temperatures throughout the study period. Oppositely, the YFPs' building walls and roof were covered by large windows, therefore exposing the animals to natural light, and the water temperature was also varied depending on the external, ambient air temperature (from 10 °C in the winter to 27 °C in the summer).

Diurnal variation in circulating GC concentrations has been demonstrated in odontocetes (killer whale: 26; BD: 41; beluga: 23). However, it has been suggested that measurements of fecal steroid metabolites may reflect the cumulative secretion and subsequent elimination of these hormones through the intestine over several hours and, as a result, may be less influenced by circadian rhythms compared to circulating steroids [71]. Despite this hypothesis and similar to circulating GCs in other odontocetes, we found an influence of diurnal variation on FGCM concentrations in the YFP, with significantly increased concentrations during the morning, supporting the concept that the YFP, like other cetaceans, also experiences diurnal secretory patterns of GCs and also indicates that cetacean fecal samples that will be used for hormonal analysis should be collected at the same time every day or should be balanced between different sampling times to avoid this bias.

We observed decreased FGCM concentrations in both the YFP and BD in association with animal enrichment. To our knowledge, this is the first report of FGCMs and their association with enrichment in cetaceans. In support of our results, lower FGCM concentrations during enrichment compared to absence of enrichment have been reported in the giant panda (*Ailuropoda melanoleuca*, [72]). Moreover, enrichment devices, including toys, have been shown to diversify captive odontocete behavior and reduce undesired behaviors [73]. Enrichment with toys has been shown to increase both object and social play in BDs, which is theoretically believed to be a reflection of good welfare and, subsequently, lower stress states [74].

A past study has found no significant influence of social group size on BD FGCM concentrations [41]. Conversely, social separation appeared to influence YFP behavior. Separated animals have been observed spending prolonged and/or frequent periods of time in front of gates, either interacting with individuals on the other side or looking at them, increasing circular swimming patterns and being less playful [75,76]. Our observations of higher FGCM concentrations associated with separation in the YFP support the theory that these behavioral changes may be the result of a stress response toward a negative stimulus and that, as is currently practiced within marine mammal facilities, these situations should be minimized. However, because BDs did not exhibit the same FGCM concentrations pattern associated with social grouping, we suggest that different species might react or adapt differently to social groupings [77], and that separation of animals may sometimes be neutral or positive when social groups appear to negatively influence behavior or FGCM concentrations [47].

To our knowledge, the influence of visitor presence on GCs has never been studied in captive odontocetes. Interestingly, in both YFP and BDs, there was an association of number of visitors and FGCM concentrations. For YFPs, there appeared to be a U-shaped relationship between FGCMs and visitor numbers, where FGCM concentrations were the lowest when few visitors were present. For BDs, the pattern was sinusoidal, with the FGCM concentrations at their lowest either in the absence of visitors or when the numbers of visitors was moderate (three to six rows) but were higher when few (one to two rows) or many visitors (more than six rows) were present. Some studies conducted in terrestrial species have found an association of higher FGCM concentrations when animals were kept in enclosures with a higher exposure to or increased numbers of visitors (black rhinos, *Diceros bicornis*, [78]; Mexican wolves, *Canis lupus baileyi* [79]; and Indian blackbucks, *Antelope cervicapra L.* [80]). In the YFP facility, smaller groups of visitors usually consisted of employees from the research base that were quiet and did not stay for long periods (usually 5 to 10 min). Conversely, larger groups were often noisier, stayed longer (up to 40 min) and often were closer to the pool as well. However, we also observed an association of higher FGCM concentrations in the absence of visitors. This suggests human presence may be enriching for this population of captive cetaceans when it is in smaller amounts [81,82].

The observation that YFPs often interacted with small groups of visitors through underwater windows or observed them from the surface corroborates this hypothesis. However, we urge caution interpreting these results because this is only a preliminary investigation of FGCMs in YFP, and we do not know if the differences we observed are within the normal HPA axis modulatory range for this species or are solely the result of visitor presence. Further study with more individuals could help clarify this question. Regarding BDs, the observed sinusoidal pattern is hard to explain, and we suggest that the parameter we used (i.e., number of visitors) might not be suitable to investigate the influence of visitors. The noise (i.e., decibel level) produced by the visitors or their activity may be more important variables and should be included in further studies.

The present study's results and their interpretation are limited by the small number of animals we sampled and by balancing issues concerning the number of samples for each individual and for each condition. Furthermore, the time between HPA activation, an increase in GC production and subsequent excretion via feces is species dependent [83,84]. The lag time from steroid hormone production to excretion in the YFP is unknown. However, recent work has provided evidence that acute stress can be detected in FGCM concentrations within 3.5 to 5 h post the acute stress event [6]. This lag time is in line with the presumed time for the passage of feces following feeding, which is around 6 h in dolphins [85]. The evidence presented within our research, where we found an influence of morning events on afternoon FGCM concentrations, between 4 and 12 h afterwards, corroborates the lag time detected by Champagne et al. [6]. Furthermore, even though we found evidence of diurnal variation in FGCMs in the YFP, FGCMs would be expected to be either lower or no different than the morning FGCM concentrations. Our observations of higher FGCM concentrations in the afternoon following unusual social events in the morning suggest influence due to diurnal variation was minimized or masked during these incidences. Although we analyzed the influence of the different conditions on the FGCM concentrations of all individuals in each species together, individuals may respond differently to certain conditions [45,86,87].

Even though the GCs are often referred to as a "stress hormone" and high concentrations can be interpreted as being reflective of distress, this hormone is part of a complex allostatic mechanism, and its link with stress is not direct and inferences of FGCMs to stress may be inappropriate [3,49]. Furthermore, associations of distress with elevated GC concentrations do not mean that the inverse assumption that elevated GC concentrations is indicative of distress is true. For instance, GCs play a major role in glucose homeostasis by promoting the genesis and storage of glucose to enhance brain function (for a review, see [88]). Short-term increases in FGCM concentrations are not necessarily linked to negative states but reflect physiological responses to various kinds of stimuli. Conversely, prolonged increased FGCM concentrations may be indicative of chronic stress [18]. Other parameters, such as immunoglobulin-A or hematology and serum chemistry analysis, in combination with GCs, may be better suited to identify distress [48,89]. Stimuli that activate a GC response are not always negative, and unless the concentration changes in the GCs are paired with some behavioral or situational context, they cannot be used to differentiate between positive stimuli and negative stress states [3]. We did not include analysis of circulating GC concentrations in our experimental design and circulating GC data may have provided more support for our FGCM results and provided further biological validation and relevance. Future study investigating the relationship between circulating GCs and their excreted metabolites is warranted.

5. Conclusions

In this study, we showed that reproductive status, physical, contextual and social variables may influence the FGCM concentrations in both YFPs and BDs. These influences can be either positive or negative. Short-term events, such as toy availability (enrichment), as well as social grouping and presence of visitors were associated with discrete changes in FGCM concentrations. Because of our small sample size, our results alone did not allow us to validate that the FGCM concentrations changes can be used to reflect acute changes in the animals' environment. However, the patterns we found

during pregnancy were consistent with that which is observed in other cetaceans and provided biologic evidence for the effectiveness of the assay system. With a valid assay system, more studies on larger sample sizes should be conducted to determine if such relationships exist. Detecting health or welfare issues in captive odontocetes can be challenging because conditions such as weight loss, reduced appetite or lethargy may not present until late in a disease process [47]. Therefore, monitoring allostatic changes via FGCM analysis in cetaceans in combination with physiological and behavioral monitoring may allow for the identification of potential stressors that can then be minimized or removed as a preventative measure. However, before inferences of FGCM concentrations can be made with respect to animal welfare, basic information, such as the circulatory and excretory dynamics, effects of sex, season, diurnal variation and age, must be examined first.

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