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Exploration of Fecal Glucocorticoid Metabolites in the Bottlenose Dolphin (*Tursiops truncatus*) Under Human Care by Enzyme Immunoassay

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Abstract

While the bottlenose dolphin (*Tursiops truncatus*) is the most studied and widespread cetacean in captivity, studies assessing its welfare have been developed only recently. Several studies focus on a potential indicator of stress: fecal glucocorticoid metabolites (FGM) concentration, which is expected to increase when the hypothalamic-pituitary-adrenal (HPA) axis is activated. However, a lack of studies that consider the biological variation of FGM concentrations related to sex and seasons, and the link to dolphins' reproductive status still impairs our ability to use it for monitoring purposes in the context of welfare assessment. Therefore, it is essential to explore and assess the potential influence of these factors on the variation of FGM concentrations in dolphins. In parallel, non-invasive sampling and methods of measurement should be developed. Thus, the authors performed a methodological validation of enzyme immunoassay (EIA) for FGM in bottlenose dolphins and studied the influence of reproductive status, sex, and seasons on FGM concentrations. The findings showed significantly higher FGM concentrations in pregnant females than in other dolphins, and significantly higher FGM concentrations in males than in nonpregnant females. Moreover, only males showed higher concentrations during spring than during autumn and winter. In parallel, megestrol acetate used for male contraception and pregnancy in females appeared to inhibit and stimulate the HPA axis, respectively. This study creates new opportunities to use EIA as a tool for monitoring FGM concentrations in dolphins and provides new data on its biological variations.

Key Words: stress, cortisol, feces, reproduction, animal welfare, cetacean, marine mammal

Introduction

Animal welfare is a major concern for zoos, including dolphinaria, recently enhanced by societal demands, which have increased their interest in this topic (Mellor et al., 2015; Clegg et al., 2017a). Therefore, dolphinaria staff need to objectively evaluate the welfare of bottlenose dolphins (*Tursiops truncatus*) in addition to their good husbandry practices. However, reliable and validated indicators of welfare are scarce. In the literature, welfare assessment criteria rely on two types of measures: (1) resource-based measures, assessing the quality of the environment; and (2) animal-based measures, directly assessing physiological and behavioral indicators (Whay et al., 2003; Whitham & Wielebnowski, 2009; Wolfensohn et al., 2018). Over the last few years, several studies have focused on the development and validation of dolphin welfare parameters using three main parameters: (1) behavior, (2) cognition, and (3) physiology (Clegg et al., 2017a). In parallel, for non-invasive studies on the physiology of wild animals, fecal hormone analyses have become a widely used method and have been developed for several species (Wasser et al., 2000; Young et al., 2004; Dantzer et al., 2010; Romano et al., 2010; Burgess et al., 2017). In particular, fecal glucocorticoid metabolites (FGM) analysis was proposed to be useful for monitoring adrenal activity and stress response in dolphins under human care (Biancani et al., 2017).

The stress response, linked to the concept of allostasis, corresponds to "the process of maintaining

stability (homeostasis) through change in both environmental stimuli and physiological mechanisms" (Romero et al., 2009, p. 376). Bottlenose dolphins, and more generally cetaceans, have to cope with predictable and unpredictable environmental stressors, both in the wild (Curry, 1999; Fair & Becker, 2000; Ayres et al., 2012; Rolland et al., 2012; Balmer et al., 2017) and in captivity (Frohoff & Packard, 1995; Morgan & Tromborg, 2007). These stressors trigger the stress response that employs physiological mediators such as the activation of the hypothalamic-pituitary-adrenal (HPA) axis to restore homeostasis. First, the hypothalamus secretes corticotropin-releasing hormone (CRH) in reaction to external or internal stimuli. Then, CRH enhances the secretion of adrenocorticotropic hormone (ACTH) by the anterior pituitary gland, which stimulates adrenal glands and their production of glucocorticoids (GC), like cortisol, the main GC in mammals, including the bottlenose dolphin (Crocker, 2018). Circulating cortisol is metabolized in the liver and excreted by the urinary tract and the digestive tract via bile. Cortisol and its metabolites are found in various body substrates, such as blood, saliva, urine, and feces, where they can be assayed (Cook, 2012; Atkinson et al., 2015). Feces represent an interesting sampling matrix because their collection requires a minimally invasive method.

Although acute activation of the HPA axis is essential for restoring homeostasis, chronic activation can lead to deleterious consequences on the organism by prolonging the catabolic, immunosuppressive, and anti-reproductive effects of GC. For example, it is well documented that when GC secretion increases, reproductive functions are impaired (Tsigos & Chrousos, 2002; Geraghty & Kaufer, 2015). Hence, GC are considered to be markers of adrenal activity and, thus, as potential stress indicators (Möstl & Palme, 2002). However, an increase in GC secretion may not be specific to stress as it can reportedly occur in dolphins in response to physiological events such as pregnancy (Steinman et al., 2016). It is therefore essential to explore the possible influence of physiological factors, such as reproductive status, on the variation of FGM concentrations in dolphins to assess the reliability and the specificity of this potential welfare parameter in dolphins.

Until now, radioimmunoassay (RIA) was used to assay cortisol concentration in dolphin feces, requiring the use of radiation in accredited laboratories (Champagne et al., 2016, 2018; Biancani et al., 2017). However, enzyme immunoassay (EIA) can be performed in any laboratory but has not yet been validated for bottlenose dolphins. Therefore, the objectives of this study were (1) to validate an EIA to monitor FGM in the feces of bottlenose dolphins under human care, (2) to assess baseline levels of FGM, and (3) to study the effects of reproduction status and sex on their concentrations over time. To explore dolphins' physiological endocrine fluctuations, FGM concentrations were analyzed in relation to seasonal variations and reproductive status for both males and females.

Methods

Animals and Housing

Fifteen bottlenose dolphins were included in the study—nine females and six males aged 6 to 42 y old (mean ± SD = 24.8 ± 12 y). These dolphins were housed in four European facilities: Parc Astérix, France (N = 5); Boudewijn Seapark, Belgium (N = 3); Harderwijk Dolfinarium, The Netherlands (N = 4); and Kolmården Djurpark, Sweden (N = 3). As reported in Table 1, three females were in the later stages of pregnancy, from 8 to 12 mo (i.e., parturition), and two were lactating during the study. Four of nine females received a contraceptive treatment containing altrenogest (Regumate®; Intervet, Madison, NJ, USA). Two males in one facility transiently received a single oral therapy of megestrol acetate (MA) to control their reproductive behavior because of the presence of a newborn calf (Megecat®; Vetoquinol, Fort Worth, TX, USA; loading dose: 20 to 40 mg per animal, twice a day for 2 to 3 wks, followed by a weekly decrease of 5 mg of the dosage over 3 to 4 wks; MA received from 13 November 2017 to 1 January 2018, covering the second sampling period; see below). Apart from contraception, no other drugs were administered to the dolphins included in the study.

The four facilities were accredited by the European Association for Aquatic Mammals (EAAM) and presented similar husbandry management. All facilities had artificial saltwater, continuously filtered and controlled daily for water quality and temperature. The water temperature could vary between 14°C (in winter) to 26°C (in summer) for outdoor pools and between 18°C (in winter) to 22°C (in summer) for indoor pools. Dolphins were fed previously frozen fish, including capelin (*Mallotus villosus*) and herring (*Clupea harengus*). Depending on the facility, some dolphins were also fed blue whiting (*Micromesistius poutassou*), sprat (*Sprattus sprattus*), mackerel (*Scomber scombrus*), squid (*Doryteuthis opalescens*), and from time to time, fish gelatin (fish gelatin powder 200° BLOOM; Louis Francois®, Cedex 2, France). All dolphins received additional vitamins (Aquavits®; International Zoo Veterinary Group, Keighley, UK).

Table 1. Status and sampling of bottlenose dolphins (*Tursiops truncatus*) in the study. Facilities: A = Boudewijn Seapark; B = Kolmården Djurpark; C = Harderwijk Dolfinarium; and D = Parc Astérix. P1 = September 2017; P2 = from mid-November to mid-December 2017; and P3 = April 2018.

Animal	Facility	Sex	Born in captivity	Year of birth	Reproductive status			Number of assayed samples (P1/P2/P3)
					P1	P2	P3	
A1	A	F	No	~1976	Anestrus (contraceptive)‡	Anestrus (contraceptive)‡	Anestrus (contraceptive)‡	11 (5/4/2)
A2	A	F	Yes	2003	Lactating (contraceptive)‡	Anestrus (contraceptive)‡	Anestrus (contraceptive)‡	14 (7/4/3)
A3	A	M	Yes	2005	Adult	Adult	Adult	14 (7/3/4)
B1	B	F	Yes	2009	Pregnant (8-9 mo)	Pregnant (10-11 mo)	Not included¶	13 (8/5/0)
B2	B	F	Yes	2012	Prepubescent	Prepubescent	Not included¶	14 (8/6/0)
B3	B	F	Yes	1999	Pregnant (7-8 mo)	Pregnant (9-10 mo)	Not included¶	15 (10/5/0)
C1	C	M	Yes	1981	Adult	Adult	Adult	15 (4/5/6)
C2	C	M	Yes	2001	Adult	Adult	Adult	17 (3/7/7)
C3	C	M	Yes	1991	Adult	Adult	Adult	19 (7/5/7)
C4	C	F	Yes	1983	Resting	Resting	Resting	14 (5/3/6)
D1	D	M	No	~1982	Adult	Adult (contraceptive)§	Adult	28 (9/9/10)
D2	D	F	No	~1977	Pregnant (9-10 mo)	Not included¶	Lactating	10 (7/0/3)
D3	D	F	Yes	1996	Resting	Anestrus (contraceptive)‡	Anestrus (contraceptive)‡	25 (10/8/7)
D4	D	F	Yes	1999	Resting	Anestrus (contraceptive)‡	Anestrus (contraceptive)‡	21 (7/6/8)
D5	D	M	Yes	1984	Adult	Adult (contraceptive)§	Adult	25 (9/8/8)

‡Altrenogest = contraceptive for females
§Megestrol acetate was transiently administrated orally in male bottlenose dolphins to control their reproductive behavior.
¶Some dolphins could not be collected during some periods (parturition, sickness).

Sample Collection

Three sampling periods were scheduled: from 4 September 2017 to 8 October 2017 (called "P1"), from 20 November 2017 to 24 December 2017 (called "P2"), and from 2 April 2018 to 6 May 2018 (called "P3"). Each period lasted 5 wks. Dolphins were sampled in the early afternoon (between 1300 and 1500 h) twice a week during each sampling period. Thus, up to 30 samples were collected per dolphin, except for females that gave birth during the collection period (Table 1).

Feces were collected under voluntary behavior during training sessions using positive reinforcement. The collection of fecal samples was part of the medical training program, so most dolphins were trained in the procedure before this study started. Dolphins were conditioned to be in dorsal decubitus and voluntarily accepted a soft 24 Fr polyvinylchloride stomach tube (Dahlhausen, Cologne, Germany) inserted 20 to 25 cm into the anal orifice. Once inserted, feces were collected passively into the tube and placed in a 2 ml plastic vial. All fecal samples were kept frozen at -20°C until steroid extraction.

Hormone Extraction and Assay

Fecal samples were placed in an oven at 60°C for ~48 h to evaporate the water. A "vortexing (non-boiling) extraction method," described by Wasser et al. (2000, p. 263), was used for hormone extraction as previously performed for fecal steroids in marine mammals (Hunt et al., 2004, 2006; Rolland et al., 2005, 2012; Champagne et al., 2016). Once dried, all samples were manually powdered and mixed in a stainless steel mortar. Approximately 75 mg of dried, well-mixed powdered feces (weighed with Sartorius CP224S; Sartorius, Goettingen, Germany; range: 74.8 to 75.6 mg) were placed in a 2 ml plastic vial containing 1 ml of 99.9% methanol, vortexed for 30 min, followed by centrifugation for 20 min

(500 g, 4°C). The supernatant (500 µl), containing GC, was transferred into a 1.5 ml plastic vial and immediately stored at -80°C until assay. Three dry fecal samples, ranging from 50 to 75 mg (67.1, 69.5, and 73.8 mg, respectively), were extracted and assayed with the same protocol, whereas six dry fecal samples that did not reach 50 mg were discarded from the study to avoid extraction bias following recommendations (Millspaugh & Washburn, 2004; Palme, 2005).

FGM concentrations were determined by using cortisol EIA detection kits (Neogen® Corporation Europe, Ayr, UK) with an assay range of 0.04 to 10 ng/ml. The assay followed the manufacturer's protocol, and a microplate reader (EMax® Plus microplate reader; Molecular Devices, Sunnyvale, CA, USA) measured the optical density (absorbance value) at 650 nm. Controls, standards, and samples were assayed in duplicate. Samples that had a coefficient of variation (CV) > 10% between duplicates were assayed again, and those that fell outside 15 to 85% on the standard curve were diluted and assayed again as well. All concentrations were expressed in ng/g of dried feces, avoiding dilution bias.

Validation Tests

Calculating intra- and inter-assay CV assessed the precision within the test. The dilution linearity was determined by serially diluting (1:2 to 1:32) fecal extracts with EIA buffer. Recovery was assessed by a spike recovery test, calculated by adding known amounts of cortisol to fecal samples. The physiological validation was assessed by MA administration, a drug known to strongly inhibit the HPA axis (Houser et al., 2017).

Data and Statistical Analyses

All concentrations were expressed with the mean ± standard deviation (SD) in ng/g of dried feces. Statistical analyses were performed using the software *SigmaPlot*, Version 12.0, and the program *R*, Version 3.4.1 (R Core Team, 2019). A difference was considered significant when the p value was less than 0.05. To test whether dolphins showed repeatable, individual-based differences within and across seasons, and whether individual data were repeatable over time (intraclass correlations and repeatabilities), linear mixed effects models (LMMs) were performed. LMMs were based on restricted maximum likelihood estimates with 1,000 permutations for p-value calculation and a LMM-based repeatability model ('rptR' package; Stoffel et al., 2018) on individual data (individual as a random factor) for all periods and separately for the three periods for both males and females.

For each individual, mean data per period were analyzed to avoid a bias linked to variations in the number of samples per individuals. Since normality was not matched for each analysis, and considering the low sample size, nonparametric tests were performed to compare differences between males and females, among females depending on their reproductive status, between individuals receiving or not receiving a contraceptive treatment, and among seasons. Kruskal-Wallis ANOVAs, with Dunn's post-hoc tests, were performed to compare female FGM concentration depending on their reproductive status, and season variations both for nonpregnant females and untreated males. Mann-Whitney tests were conducted to compare FGM concentrations of pregnant vs nonpregnant females over periods, and sex effect between pregnant females vs males and nonpregnant females vs males for each season.

Results

We assayed 255 fecal samples from 15 dolphins over three periods (Table 1). Descriptive statistics of measured FGM concentrations are reported in Table 2.

Assay Validation

As the bottlenose dolphin excretes cortisol, the native hormone in feces (Biancani et al., 2017), FGM concentration was assayed with a cortisol EIA detection kit as recommended by Cook (2012). This kit is reported to be nonspecies specific and suitable for a variety of sample matrices. The anti-cortisol antibody in this assay was raised in the rabbit and showed the following cross-reactions: cortisol, 100%; prednisolone, 47.4%; cortisone, 15.7%; 11-deoxycortisol, 15.0%; prednisone, 7.83%; corticosterone, 4.81%; 6ß-hydroxycortisol, 1.37%; 17-hydroxyprogesterone, 1.36%; deoxycorticosterone, 0.94%; and progesterone, 0.06%. Steroids with cross-reactivity under 0.06% are not listed (e.g., betamethasone, testosterone, estradiol, cholesterol, etc.).

Intra-assay and inter-assay CV were 9.8 and 14.6%, respectively. The dilution linearity showed a mean error percentage of 6.5 ± 11.0% and a coefficient of determination $R^2 = 1.0$, confirming that fecal samples interacted with the assay antibody in a dose-dependent manner and supporting the hypothesis that the antibody-binding characteristics of FGM were similar to those of the standard samples provided with the kit (Andreasson et al., 2015). The average recovery percentage from spike recovery test was 84.9 ± 16.0%, indicating that the estimation of cortisol concentration was not affected by other components of the feces extracts.

Concerning physiological validation, FGM concentrations significantly decreased after MA administration (before administration in P1: 66.9 ± 22.4 ng/g; during administration in P2: 22.0

Table 2. Descriptive statistics of fecal glucocorticoid metabolites (FGM) concentrations from 15 bottlenose dolphins over three collection periods ($N = 255$). Females in anestrus received a contraception treatment; and resting females were nonreproductive mature females. [FGM] = FGM concentration; Prepub. = prepubescent; and MA = megestrol acetate.

	[FGM] (ng/g of dried feces)						
	Females					Males	
	Pregnant	Lactating	Resting	Anestrus	Prepub.	Without MA	With MA
Minimum	158.3	24.1	21.8	15.6	34.2	21.7	16.6
1st quartile	197.2	25.0	24.3	29.8	56.8	49.7	19.3
Median	216.6	25.8	34.8	30.3	105.9	66.5	22.0
3rd quartile	250.4	26.6	42.4	32.0	126.0	99.5	24.7
Maximum	286.9	27.4	43.7	49.5	212.7	153.9	27.4
Mean	221.9	25.8	33.4	31.6	106.0	75.3	22.0
Standard deviation	49.3	2.3	10.1	8.8	57.3	36.9	7.7

± 7.7 ng/g; *significant* post-hoc tests, $p < 0.05$), revealing that measured FGM concentrations reflected circulating GC variations.

Individual-Based Differences and Repeatability of FGM Concentrations

Taking data from both sexes across the whole study period, individual FGM values were significantly repeatable: significant individual differences were observed across time (LMM-based intraclass correlation with 1,000 permutations: repeatability intraclass correlation [RICC] = 0.626, $p < 0.001$). Therefore, individuals showed significant, individually repeatable FGM values across time. Individual FGM concentrations during P1 showed significant repeatability for females (RICC = 0.730, $p < 0.001$) but not for males (RICC = 0.038, $p = 0.281$) when considering periods and sex separately. During P2, both females and males showed significant stable concentrations (RICC = 0.761, $p < 0.001$; and RICC = 0.466, $p = 0.004$, respectively). In season P3, individual FGM concentrations showed significant repeatability for males (RICC = 0.233, $p = 0.016$) but not for females (RICC = 0.034, $p = 0.339$).

Effect of Female Reproductive Status on FGM Concentrations

Females were distinguished according to their reproductive status: prepubescent, pregnant, lactating, in continuous anestrus due to contraception (called "females in anestrus"), and nonreproductive mature (called "resting females"). Over the three periods studied, female bottlenose dolphins of different reproductive status had significantly different FGM concentrations (Figure 1; H = 15.40, $df = 4$, $p = 0.004$).

Pregnant females had significantly higher FGM concentrations (221.9 ± 49.3 ng/g; $N = 3$) than lactating females (25.8 ± 2.3 ng/g; $N = 2$) and females in anestrus (31.6 ± 8.8 ng/g; $N = 4$; *significant* post-hoc tests, $p < 0.05$). Although resting females had lower values (33.4 ± 10.1 ng/g; $N = 3$) than pregnant females, the difference was not significant, likely because of a lack of statistical power. The prepubescent female, B2, exhibited intermediate concentrations (106 ± 57.3 ng/g; $N = 1$), while FGM concentrations in resting females, females in anestrus, and lactating females exhibited FGM values in a similar range that did not differ significantly (nonsignificant post-hoc tests, $p > 0.05$).

Then, mature females were distinguished according to their pregnancy status: (1) pregnant females and (2) nonpregnant females, including resting females, females in anestrus, and lactating females. These two groups exhibited significantly different FGM concentrations (Figure 2A; U = 95; $n = 5$ and $n = 16$, respectively; $p = 0.001$) with a mean value of 31.4 ± 8.6 ng/g for nonpregnant females, which is significantly lower than pregnant females.

Effect of Contraception on FGM Concentrations

As stated above, no significant difference was observed between resting females and females in anestrus. However, the two males that received megestrol acetate (MA) from the beginning of the P2 period showed a decrease in FGM concentrations of approximately 70% between P1 and P2, while untreated males showed an increase of approximately 50 to 60% for two individuals and a decrease of approximately 40 to 50% for the other two. In P2, treated males had mean FGM concentrations of 22.0 ± 7.7 ng/g (vs 66.9 ± 22.4 ng/g in P1), while untreated males had a mean concentration of 53.6 ± 29.4 ng/g (vs 50.8 ± 9.1 ng/g in P1; Figure 2B).

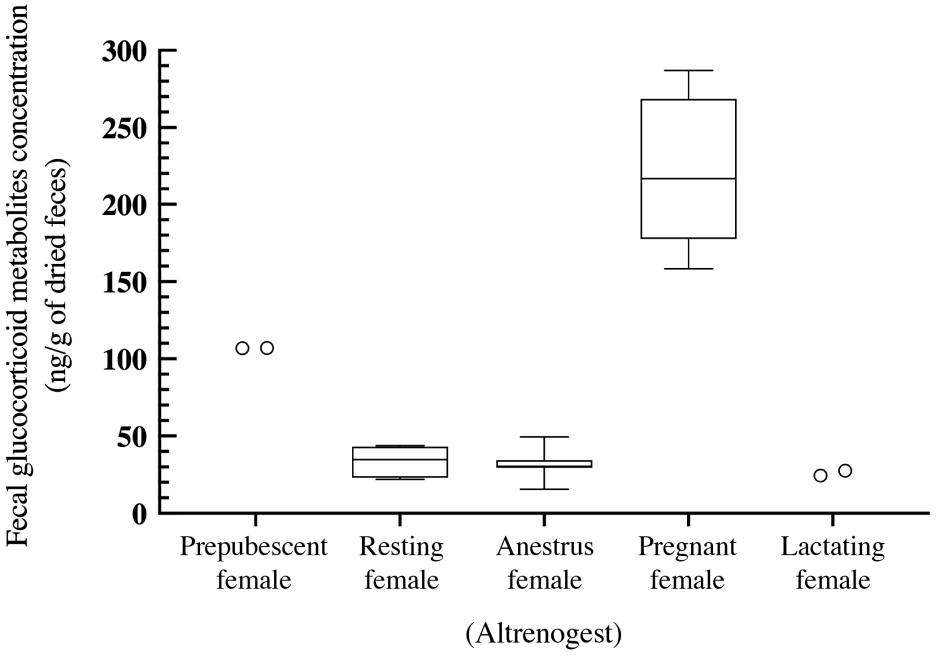

Figure 1. Boxplots of fecal glucocorticoid metabolites (FGM) concentrations of female bottlenose dolphins (*Tursiops truncatus*) of different reproductive status. The line within the box is the median, the box encloses data between the first and the third quartile, and the whiskers outside the box represent the minimum and the maximum. When number of values was less than 3, values were put directly on the graphic (circles). Note that one of the two lactating females was receiving altrenogest contraceptive.

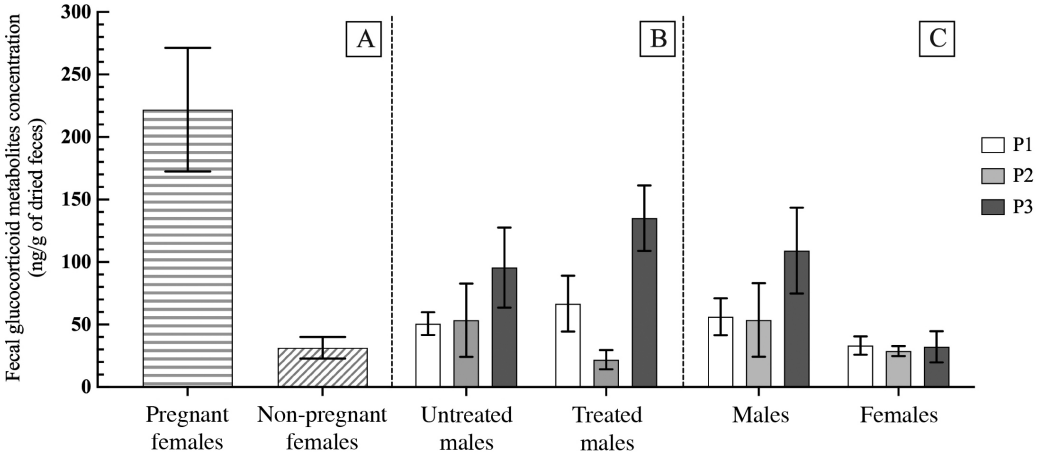

Figure 2. FGM concentrations (mean ± SD) in female bottlenose dolphins according to pregnancy status (A), in male bottlenose dolphins treated or not treated with megestrol acetate (MA) in P2 (B), and in male and nonpregnant female bottlenose dolphins across time (C). Nonpregnant females include resting females (i.e., nonreproductive mature females), females in anestrus because of altrenogest treatment, and lactating females. Values in P2 from males treated with MA are not included in Figure 2C. P1 = September 2017; P2 = from mid-November to mid-December 2017; and P3 = April 2018.

Because of the observed diminution of FGM concentrations in P2 for the two males treated with MA, the authors decided not to use their data in P2 for further statistical analyses. Given the 3-mo interval between the end of MA administration and P3, a rebound effect in FGM concentrations after MA withdrawal was unlikely, and P3 values were thus considered for these two males.

Effect of Sex on FGM Concentrations

Over the entire study period, pregnant females had significantly higher FGM concentrations than males (75.3 ± 36.9 ng/g; U = 95; n = 5 and n = 16, respectively; p = 0.001), and males had significantly higher FGM concentrations than nonpregnant females (U = 369; n = 16 and n = 16, respectively; $p < 0.001$).

Effect of Seasonal Periods on FGM Concentrations

No significant difference was observed among the different periods for nonpregnant females (Figure 2C; $H = 0.534$, $df = 2$, $p = 0.766$). However, male bottlenose dolphins had significantly different concentrations among periods (H = 6.812, df = 2, p = 0.033). Though post-hoc tests did not show any significant difference, FGM concentrations tended to be higher in P3 than in P1 and P2 (Figure 2C; mean difference = 52.8 ng/g P3 vs P1; mean difference = 55.4 ng/g P3 vs P2).

Among males, two individuals (C1 and C2) were kept in a male group while the others were in mixed groups, yet all males showed the same range and evolution of FGM over the study (Figure 3). Moreover, one male (C3) lived in an outdoor lagoon within a mixed group and did not participate in public presentations. The same range and evolution of his FGM concentrations were observed as they were for the other males who were involved in public presentations, with higher FGM concentration in P3 (94.2 ± 56.3 ng/g) than in P1 (46.0 ± 17.0 ng/g) or in P2 (72.3 ± 52.6 ng/g).

Discussion

In the actual holistic framework of dolphin welfare assessment, it is suggested to use behavioral, cognitive, and physiological indicators (Webster, 2005), and cortisol measures have been one

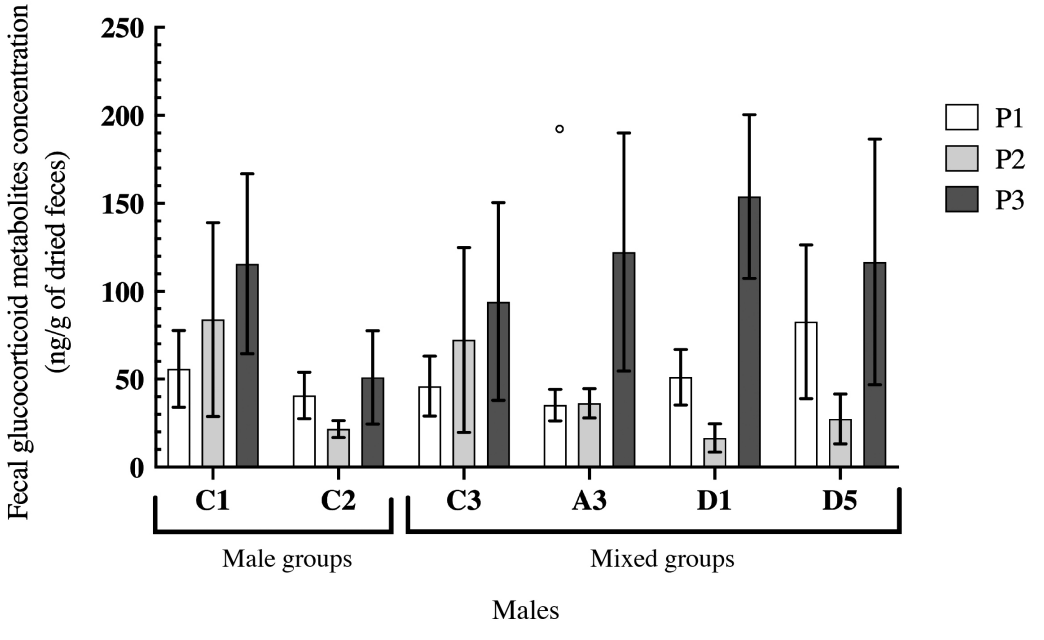

Figure 3. Individual FGM concentration in male bottlenose dolphins (mean ± SD). Dolphins C1 and C2 evolved exclusively in a group of male dolphins whereas the other males belonged to mixed groups including males and females. P1 = September 2017; P2 = from mid-November to mid-December 2017; and P3 = April 2018. Note the outlier value from the male A3 during P1.

criterion to assess. The findings of this study on the assessment of FGM concentrations using EIA proved its feasibility. The authors suggest implementing this parameter in future dolphin welfare assessment. However, the observed variations suggest careful consideration of bottlenose dolphins' sex and reproductive status if using FGM measurement to evaluate potential exposition of dolphins to stress. The authors propose that convergent data from several physiological, behavioral, and cognitive indicators should be used in a holistic approach if aiming to evaluate dolphins' welfare. Moreover, this study did not aim to validate FGM concentrations as a welfare parameter but to perform a validation of EIA for FGM in bottlenose dolphins and to monitor its physiological variations.

EIA Validation

Methodological validation of the EIA kit confirmed the reliability of the measured concentrations. Good parallelism and linearity of the dilution were obtained, as well as satisfying recovery, intra-assay, and inter-assay precisions.

The physiological validation confirmed the relevance of the EIA kit to detect biologically significant alteration in adrenocortical activity. Physiological validation requires a study to "pharmacologically induce physiological changes in circulating GC levels and to evaluate whether these changes are reflected in measured concentrations of fecal [GC metabolites] afterward" (Touma & Palme, 2005, p. 62). Given regulatory and ethical constraints, an ACTH challenge test or a dexamethasone-induced suppression test were not possible in the context of this study. However, MA administration was reported to strongly suppress cortisol secretion in male dolphins (Houser et al., 2017) and, thus, could represent a relevant physiological validation approach. The observed decrease in FGM concentrations for treated males over the considered period might reflect HPA axis inhibition by MA, although a larger number of individuals would be required for definitive conclusion. Biological validation is an alternative to physiological validation and is based on the physiological stress response naturally induced by a stressor such as transportation to another facility. No such opportunity arose during this study; however, results suggested a possible biological validation. Indeed, pregnant females had a FGM concentration significantly higher than other dolphins in accordance with a previous report indicating that circulating cortisol concentration increased during pregnancy, peaking in the late stage (Steinman et al., 2016). Thus, this result could stand for a biological validation with physiological stress induced by pregnancy.

The performance of this EIA kit, therefore, seemed suitable for the measurement of FGM concentrations in bottlenose dolphin fecal samples and could be an alternative to RIA. Further investigations could be interesting to perform to confirm the biological validation, with another stressful event such as transportation, or the physiological validation, with a larger sample size.

The small sample size of this study was a limit for the interpretation of some results. For example, the prepubescent female's results (N = 1) provided an estimate in FGM concentrations for young immature females but did not allow a statistical comparison with other bottlenose dolphins. For other subsamples, like lactating females and treated males, the number of individuals was small (N = 2), so a larger subsample size would be required for definitive conclusions. Although the sample size was small, the number of 15 dolphins was relatively high compared to other studies in marine mammals (Biancani et al., 2009, 2017; Houser et al., 2011, 2017; Clegg et al., 2017b).

Intra- and Inter-Individual Variability

Variability could be observed for FGM concentrations from samples both at the intra- and inter-individual level. Because dolphins' feces are liquid, collection did not involve the whole fecal mass but a sample of it. FGM might not be evenly distributed among feces (Millspaugh & Washburn, 2003), and this might contribute to intra-individual variations. In parallel, variable transit time may also account for non-even distribution of FGM among feces (Millspaugh & Washburn, 2003), and secretion of cortisol may follow circadian variations as described for other cetaceans (Suzuki et al., 2003; Schmitt et al., 2010). In our study, fecal samples were collected in the early afternoon at each session so potential influence of circadian rhythm of cortisol secretion and transit time on FGM concentrations was maintained as low as possible.

Potential influence of environmental factors on adrenocortical activity was avoided during the study to measure FGM baseline levels. No stressful events occurred during sampling periods such as transportation, capture, noisy handling works (e.g., repair/reconstruction work), etc. Water temperature is an environmental factor that can interact in adrenocortical activity. Cold stress stimulated the HPA axis, leading to an elevation of circulating GC (Houser et al., 2011). In this study, water temperature was controlled daily and could vary between 14°C (in winter) to 26°C (in summer) for outdoor pools and between 18°C (in winter) to 22°C (in summer) for indoor pools. Since water temperature remained stable and did not drop below 14°C, water temperature was not considered as a variable that could affect adrenocortical activity and, by extension, FGM concentrations.

Finally, individual FGM values were significantly repeatable considering the three periods, with significant individual differences (LMM-based intraclass correlations), which indicates that the putative causes of intra-individual variations did not prevent from discriminating inter-individual causes of variation.

Physiological Variations According to Sex, Reproductive Status, and Seasons

This study reported a statistical difference in FGM concentration between sexes: pregnant females had significantly higher FGM concentrations than males without contraceptive treatment who, in turn, had significantly higher FGM concentrations than nonpregnant females. While sex differences had not previously been observed in bottlenose dolphins (Biancani et al., 2017), they had been reported in other marine mammals such as the North Atlantic right whale (*Eubalaena glacialis*) and the Steller sea lion (*Eumetopias jubatus*) (Hunt et al., 2004, 2006).

Seasonal variation in cortisol is still controversial in bottlenose dolphins. According to Orlov et al. (1988), serum cortisol may be higher in winter and spring than in summer and autumn, yet St Aubin et al. (1996) did not observe any significant seasonal variation. Regarding FGM, Biancani et al. (2017) did not demonstrate any seasonal change contrary to this study which showed a higher concentration in spring (P3). This period corresponded to the reopening of facilities to visitors after the winter break and resumption of public presentations. Nevertheless, FGM increase in P3 was not observed in females, suggesting a sexual origin in males rather than an unlikely sex-restricted stress. This hypothesis is corroborated by the fact that one male (C3) who lived in a lagoon where no public presentation was performed had a similar variation of FGM concentrations to other males. This result suggested that resumption of public presentations did not influence FGM concentrations in bottlenose dolphins under human care in our conditions. It should be noted that summer was not included in our study and that samples were collected only over three periods of 5 wks. Additional studies, including a larger sample size with fecal sample collection distributed evenly over a full year, could bring complementary information on seasonal variation of FGM concentrations.

Physiological elevation of FGM in male dolphins in spring might reflect roles for endogenous GC in reproduction. Whereas an inhibitory role of abnormally elevated GC on testis function is well documented in mammals (Sapolsky et al., 2000; Geraghty & Kaufer, 2015), for example, with Cushing's disease, severe stress, or exposure to pharmacological GC, a stimulating role of physiological levels of GC for testis function has been identified more recently (Whirledge & Cidlowski, 2010). In particular, adrenalectomy impairs testicular structure and spermatogenesis (Silva et al., 2014), as well as inactivation of GC receptor in Sertoli cells (Hazra et al., 2014). Precise dosage of endogenous GC is thus necessary for proper testis function (Suarez et al., 2012), and further investigations would help in deciphering seasonal influence of GC concentration for male dolphins' fertility.

Conclusion

In conclusion, this study validated a FGM measurement by EIA in bottlenose dolphins and accumulated evidence that FGM concentrations were modulated by sex and reproductive status of dolphins. Pregnancy markedly increased FGM concentrations whereas MA decreased FGM concentrations in males. The observed variations of adrenal activity according to the reproductive status of bottlenose dolphins under human care might suggest that the endocrine function in studied dolphins was not impaired by chronic stress. However, the observed variations lead us to suggest careful consideration of bottlenose dolphins' sex and reproductive status if using FGM measurements to evaluate potential exposition of dolphins to stress. We propose that convergent data from several physiological, cognitive, and behavioral indicators should be used in a holistic approach if aiming to evaluate dolphins' welfare.

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