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PHYSIOLOGICAL ECOLOGY – ORIGINAL RESEARCH

Potential endocrine correlation with exposure to domoic acid in Southern Right Whale (*Eubalaena australis*) at the Península Valdés breeding ground

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Abstract

In waters off Península Valdés (PV), Argentina, southern right whales (SRW, *Eubalaena australis*) are occasionally exposed to domoic acid (DA), a neurotoxin produced by diatoms of the genus *Pseudo-nitzschia*. Domoic acid toxicity in marine mammals can cause gastrointestinal and neurological clinical signs, alterations in hematologic and endocrine variables, and can be fatal in extreme cases. In this study, we validated an enzyme immunoassay to quantify fecal glucocorticoid metabolites (fGCm) in 16 SRW fecal samples from live and dead stranded whales in PV from 2013 to 2018 and assessed fGCm levels associated with DA exposure. Overall, fGCm levels were significantly lower in SRWs with detectable fecal DA ($n=3$) as compared to SRWs with undetectable fecal DA levels ($n=13$). The highest fecal DA was observed in a live lactating female, which had low fGCm compared to the other lactating females studied. The highest fGCm was observed in a lactating female with undetectable DA; interestingly, at the time of sample collection, this female was sighted with two calves, an extremely unusual occurrence in this species. Though the sample size of these exceptionally rare breeding-season fecal samples was unavoidably small, our study provides evidence of potential adrenal alterations in whales exposed to an environmental neurotoxin such as DA.

Keywords Fecal hormones · Glucocorticoids · Phycotoxin · Wildlife health · Validations

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Introduction

The phycotoxin domoic acid (DA) is a potent water-soluble neurotoxin naturally synthesized by several species of diatom of the genus *Pseudo-nitzschia*. In vertebrates, including humans, DA ingestion can cause gastrointestinal and neurological clinical signs that can result in death (Perl et al. 1990; Silvagni et al. 2005; Pulido 2008). Domoic acid exposure from harmful algal blooms (HABs) thus poses a risk to the safety and health of humans and wildlife. This neurotoxin has caused die-offs in many marine mammal species, including both pinnipeds and cetaceans (Gulland 1999; Lefebvre et al. 1999; Fire et al. 2010, 2021; Broadwater et al. 2018). Chronic exposure of marine mammals to DA can also cause sublethal effects, including degenerative heart disease, chronic epileptic syndromes, reproductive failure, and altered hematology and endocrinology (Scholin et al. 2000; Brodie et al. 2006; Goldstein et al. 2009; Zabka et al. 2009; Gulland et al. 2012).

Chronic or acute exposure to DA constitutes a stressor that may affect glucocorticoid concentrations. The glucocorticoids (GCs; cortisol and corticosterone) are adrenal steroid hormones that maintain essential functions of metabolism and energy balance, and that increase sharply in response to environmental stressors (Sapolsky et al. 2000; Bornier et al. 2009). Synthesis and secretion of GCs is controlled by the hypothalamus–pituitary–adrenal (HPA) axis. In most vertebrates, a variety of stressors, including malnutrition, predation, harassment, and injury, can elevate GCs (Romero and Wingfield 2016), which then elicit a variety of adaptive physiological and behavioral responses (McEwen and Wingfield 2003; French et al. 2007; Romero et al. 2009; Meylan et al. 2010). However, in chronic stress these relationships can reverse, with GCs sometimes declining, especially in moribund individuals (Dickens and Romero 2013; Fernández Ajó et al. 2018). As an additional complication, domoic acid is an excitatory amino acid analog of glutamate, a well-known brain neurotransmitter that activates glutamate receptors (Pulido 2008) and that can play an important role in the activation of the HPA axis, regulating many pituitary hormones involved in the stress response (Brann and Mahesh 1994; Johnson et al. 2001). Thus, GC levels could rise during DA exposure via two mechanisms: a generalized HPA response to the physiological stress imposed by DA-related illness, or a targeted effect of DA on pituitary hormone release. Nevertheless, studies have reported the opposite correlation, detecting lower serum cortisol levels in California sea lions (*Zalophus californianus*) exposed to DA than in unexposed animals. This suggests that exposure to DA could lead to an adrenal function insufficiency (Gulland et al. 2012). Overall, GC levels are a potential useful metric for assessing the physiological impacts of exposure to DA.

With the global increase in magnitude and frequency of HABs associated with ocean warming (Moore et al. 2008; Van Dolah 2000), DA poisoning represents a significant health risk for marine mammals, with potential endocrine effects. Thus, more studies are needed to clarify the nature of any relationship between DA exposure and GC levels in marine mammals. Glucocorticoids are traditionally measured in plasma, but plasma sampling from free-ranging large whales is currently impossible. However, alternative sample types such as fecal samples, baleen, respiratory vapor, and blubber can be utilized to quantify GCs in large whales (Rolland et al. 2005, 2017; Hunt et al. 2006, 2014, 2019; Hogg et al. 2009; Fernández Ajó et al. 2020). The analyses of fecal glucocorticoid metabolites (fGCm) have proven particularly useful for endocrine assessments of free-swimming whales, with several studies showing that fGCm correlate in meaningful ways with presumed stressors. For example, high levels of fGCm in North Atlantic right whales (NARW, *Eubalaena glacialis*) correlate with poor body condition and entanglements (Hunt et al. 2006). A significant reduction in ambient noise was associated with decreased fGCm in NARW (Rolland et al. 2012), and fGCm increases were associated with entanglements and ship strikes (i.e., Rolland et al. 2017; Lemos et al. 2020). Results from fGCm analysis can guide management and conservation actions by distinguishing the relative importance of different stressors (Ayres et al. 2012). However, the extraction and analysis of fecal hormones require both biological and analytical validations when applying to a new species.

The southwestern Atlantic southern right whale (SRW, *E. australis*) population that breeds off Península Valdés, Argentina, migrates annually from their feeding ground and remains in the region of Península Valdés during the austral winter and spring months. In Península Valdés, SRW individuals gather to mate, give birth, and nurse their calves (Bastida and Rodríguez, 2009). The peak in whale abundance occurs from August through September (Crespo et al. 2019). By mid-December, almost all individuals have left Península Valdés to summer at feeding grounds at mid and high latitudes of the South Atlantic and sub-Antarctic regions (Rowntree et al. 2008; Valenzuela et al. 2009), with some individuals moving east of Península Valdés to forage in the offshore Península Valdés front (Zerbini et al. 2018).

Southern right whales are capital breeders, largely fasting during the breeding season and instead relying on stored blubber fuel reserves. Thus, Península Valdés is not considered a feeding ground. However, adults and juveniles do occasionally feed in the gulfs of the Península during spring, mainly on calanoid copepods (Hoffmeyer et al. 2010; D'Agostino et al. 2016, 2018). Diatoms of the genus *Pseudo-nitzschia* dominate the spring phytoplankton blooms

in this area (Sastre et al. 2007; D'Agostino et al. 2015, 2018). Therefore, feeding SRWs in Península Valdés temporally overlap with these *Pseudo-nitzschia* blooms (D'Agostino et al. 2018) and represents a potential test case for assessing the relationship of DA exposure with GC levels.

Southern right whales are not exposed to as many anthropogenic stressors as their congener, the NARW (Hunt et al. 2021); however, between 2003 and 2015 an unusually large number of SRW calves died at Península Valdés, with population-level consequences (Marón et al. 2015a). These high calf mortalities prompted the Scientific Committee of the International Whaling Commission to convene two workshops to analyze mortality data, hypothesize potential causes, and identify research priorities (International Whaling Commission [IWC] 2011, 2015). Considerable research effort has focused on the analysis of these unusual mortalities (see for example, Marón et al. 2015b; McAloose et al. 2016; Fernández Ajó et al. 2018, 2020). In particular, investigation of exposure to phycotoxins has shown that SRWs are exposed to DA during their stay in the Península Valdés breeding ground (Wilson et al. 2015; D'Agostino et al. 2017); however, we are not aware of any studies aimed at understanding the possible effects of exposure to phycotoxins on whale physiology and health.

The goals of this study were to (1) detect the exposure of SRWs to DA at their breeding ground in Península Valdés, Argentina, and (2) identify potential endocrine correlates of DA exposure. To accomplish these goals, an enzyme immunoassay was validated to quantify fGCm in SRW fecal samples. Domoic acid data presented here include some data previously described in D'Agostino et al. (2017), reanalyzed here to include additional samples collected since, and with the full dataset of DA then compared to fGCm data. In the present study, all fecal samples available ($n = 16$) over a 6-year period were used; sample size is unavoidably low given that, at Península Valdés, detection and collection of fecal samples is rare. Nonetheless, we report data from these samples as preliminary assessments to identify avenues for further research.

Materials and methods

Study species

The SRW has a circumpolar distribution in the Southern Hemisphere, migrating annually between productive feeding grounds and sheltered nursery grounds. Feeding typically occurs in austral summer and fall in regions located at mid and high latitudes of the South Atlantic and sub-Antarctic (Rowntree et al. 2008; Valenzuela et al. 2009). In these areas, SRWs primarily feed on euphausiids south of 50° S, on copepods north of 40° S, and on a mixture of euphausiids and copepods between these latitudes (Tormosov et al. 1998). Calving occurs off the coasts of Argentina, Brazil, South Africa, New Zealand and Australia during austral winter and spring (IWC 2001). Birthing generally occurs between August and late October (Bastida and Rodríguez 2009). Females stay at the calving grounds with their calves for about 77 days after birth (Rowntree et al., 2001). The mother–calf pairs stay in the area longer than other groups of whales (e.g., males) and they are the last to leave the area (Rowntree et al. 2001). Single calves are the norm for this species, and twinning has never been observed. Lactation duration is poorly known for this species (Best et al. 2015). Some calves have been seen feeding on zooplankton patches next to their mothers at an estimated age of 6 months (Best 2007). In other cases, females return to coastal breeding areas in the year following birth, still accompanied by their yearling calf; therefore, if these calves are still nursing, lactation would have lasted about 12 months (Thomas and Taber 1984; Best et al. 2003, 2015).

Fecal sample collection

Sixteen fecal samples were collected from live free-swimming ($n = 13$) and deceased stranded ($n = 3$) whales in Golfo Nuevo (GN, Fig. 1) during the 2013–2018 SRW breeding seasons from July to December (Table 1). Fecal samples from live whales were collected with a hand-held 125 µm mesh net deployed from a boat. Samples from stranded individuals were collected directly from the intestine of dead whales during necropsies. Fecal samples both from live and dead animals were placed in plastic containers in coolers and frozen (− 20 °C) within the day of collection. Sex was determined either by direct observation of the shape of the genital area (Payne et al. 1983) or, in the case of lactating females, sighting the whale closely accompanied by a calf. Clearance studies for DA have not been conducted on SRWs. However, several studies have documented that DA is cleared rapidly in mammals, i.e., within 48 h of ingestion (Iverson et al. 1989; Truelove and Iverson 1994; Maucher and Ramsdell 2007; Wittmaack et al. 2015). Likewise, clearance studies for fGCm have not been conducted on SRWs. However, it is estimated that fecal hormone metabolites reflect the average level of circulating parent hormone in blood with a lag time of hours to days, depending on hormone turnover rates and gastrointestinal passage time for the species. Based on data from other species, the lag time for right whales is usually estimated at 24 h, i.e., fGCm concentrations in a given fecal sample likely reflects circulating levels in plasma of the day prior to fecal sample collection (Millspaugh and Washburn 2004; Rolland et al. 2007, 2012). These findings suggest that the SRWs analyzed in our study were exposed to DA recently and the fGCm can be correlated with the DA exposure.

Fig. 1 Study area showing the location in Golfo Nuevo, Chubut, Argentina, where southern right whale (*Eubalaena australis*) fecal samples from live and dead stranded individuals were collected (shown in blue)

Table 1 Levels of domoic acid (DA), immunoreactive fecal corticosterone metabolites and immunoreactive fecal cortisol metabolites in fecal samples of southern right whale from Golfo Nuevo (GN), Argentina. Bahía Pirámide (BP); levels of DA below detection limit is indicated with < dl; whales of unknown sex or age are reported as Unk; black stars indicate DA data reported previously by D'Agostino et al. (2017); black dot refers to a sample collected from a live lactating female sighted with two calves

Whale ID	Sample location	Date collected	State	Age class/Sex	DA [μg g^{-1}]	Corticosterone [ng g^{-1}]	Cortisol [ng g^{-1}]
BFA1★	GN	29 Jul 2013	Dead	Unk	< dl	160.66	96.28
BFA2★	BP (GN)	6 Oct 2013	Dead	Unk	< dl	56.70	4.73
BFA17	BP (GN)	25 Oct 2017	Live	Calf unk	< dl	33.58	11.98
BFA19	BP (GN)	Season 2018	Live	Calf unk	< dl	32.95	41.89
BFA6★	Playa Kaiser (GN)	5 Oct 2014	Dead	Juvenile male	< dl	10.89	3.94
BFA11★	Pta. Piaggio (GN)	11 Oct 2015	Live	Juvenile unk	1.00	69.72	26.68
BFA13★	BP (GN)	15 Nov 2015	Live	Adult unk	0.30	15.50	4.38
BFA16	BP (GN)	7 Aug 2017	Live	Adult unk	< dl	312.38	52.35
BFA4★	BP (GN)	18 Sep 2014	Live	Lactating female	< dl	95.18	22.48
BFA7★	BP (GN)	13 Oct 2014	Live	Lactating female	< dl	298.43	53.75
BFA8★	BP (GN)	17 Nov 2014	Live	Lactating female	< dl	58.60	19.62
BFA9★	BP (GN)	19 Nov 2014	Live	Lactating female	710 ± 75	19.48	7.66
BFA10★	BP (GN)	22 Nov 2014	Live	Lactating female	< dl	206.57	142.12
BFA14★	BP (GN)	15 Dec 2015	Live	Lactating female	< dl	12.38	5.38
BFA18	BP (GN)	26 Nov 2018	Live	Lactating female	< dl	72.90	20.32
BFA20●	BP (GN)	22 Dec 2018	Live	Lactating female	< dl	360.95	198.29

Detection limit ($S/N > 3$) ranges were determined between 0.01 and 0.11 μg DA g^{-1} fecal sample [dry weight] and quantification limit ($S/N > 10$) ranges between 0.03 and 0.37 μg DA g^{-1} fecal sample [dry weight] in dependence of extracted sample weight, respectively

Domoic acid determination

Southern right whale feces were lyophilized to remove water (OPERON FDU-8606, Korea) and stored at − 20 °C until DA was extracted following D'Agostino et al. (2017). Briefly, fecal samples were thawed at room temperature and ~ 10 mg aliquots (± 0.001%; BA110S, Sartorius, Göttingen, Germany) transferred to FastPrep tubes containing 0.9 g ceramic beads (lysing matrix D, Thermo Savant, Illkirch, France), and 1 ml methanol was added, homogenized by

reciprocal shaking at maximum speed (6.5 m s^{-1}) for 45 s in a Bio101 FastPrep homogenizer (Thermo Savant, Illkirch, France), and centrifuged at 16,100×*g* at 4 °C for 10 min. The supernatants were transferred to spin-filters (0.45 µm pore-size; Millipore Ultrafree, Eschborn, Germany) and centrifuged at 16,100×*g* at 4 °C for 5 min. The resulting filtrates were transferred to autosampler vials; the final volumes were adjusted to 1 ml with methanol and stored at 4 °C until use. The residues of each fecal sample in the FastPrep tubes were re-extracted once as described above. Accordingly, two extracts were obtained for each fecal sample, and both were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) separately (for protocol details of the LC–MS/MS analyses, see D'Agostino et al. 2017). One sample (BFA9, see Table 1) presented high values of DA that fell outside the calibration range. This sample was divided into three aliquots, with each consecutively extracted 20 times ($n = 60$). Most DA in this sample was determined in the first two extracts, with the 3rd–20th extractions yielding only minor additional DA (38% of the sum of the first two extracts). To avoid the very time-intensive multiple extractions, the two other samples (BFA11, BFA13) with high DA levels were only extracted twice, with final DA content estimated as the sum of DA in the first extract and second extract, plus a correction factor of 0.38 based on data from the 3rd–20th extractions of sample BFA9. Domoic acid levels were expressed as µg g^{-1} dry fecal sample.

Hormone quantifications

Fecal sample preparation

Laboratory hormone extraction and analyses were performed at Northern Arizona University. Freeze-dried fecal samples were homogenized in individual glass vials by thoroughly stirring with a metal rod for ≥ 1 min before weighing. For each sample, 100 mg of fecal powder was weighed using a digital scale (± 0.0001 g; Ohaus Explorer Pro EP214C, Pine Brook, NJ, USA). To reduce static electric discharge when processing fecal powder, a workstation ionizer (SPI No. 94000, SPIwesstek.com) was activated next to the digital scale whenever fecal powder was handled or weighed. To avoid cross-contamination, gloves were changed between samples, and the work area and equipment were comprehensively cleaned with 70% ethanol. Weighed fecal samples were placed in 16 × 100 mm borosilicate glass tubes and sealed until extraction; all extractions were performed within 24 h after aliquoting.

Steroid hormone extraction

100 mg of well-mixed fecal powder was combined with 6.00 ml 100% methanol (HPLC grade, Thermo Fisher Scientific), vortexed for 2 h at room temperature (Large Capacity Mixer, Glas-Col, Terre Haute, IN, USA; speed set on 40), and centrifuged for 1 min at 4025×*g*, after which the supernatant was transferred to a 13 × 100 mm borosilicate glass tube. The supernatant (1.00 ml) was extracted using solid-phase extraction (SPE, detailed in Newman et al. 2008) to reduce matrix effects (see validations below). Samples were then eluted in 90% methanol (10% distilled water), dried in a ThermoSavant SpeedVac Concentrator (model SDP121P; Thermo Fisher Scientific, Waltham, MA, USA) at 35 °C, reconstituted in 0.50 ml assay buffer (X065 buffer; Arbor Assays, Ann Arbor, MI, USA), bath sonicated for 15 min (Branson 3800 ultrasonic cleaner), vortexed for 15 min, transferred to 1.5 ml vapor proof o-ring-capped cryovials, and stored at − 80 °C until assay within 1 week. Percentage recovery was not evaluated in this study, as it is not possible to mimic behavior of native hormone in non-plasma sample types via addition of liquid radiolabelled parent hormone, particularly given that fecal hormone metabolites are not chemically identical to the parent hormone (Palme et al. 2013); rather, data analysis focuses on relative patterns and not on absolute concentrations.

Hormone assays and validation

Commercial enzyme immunoassay (EIA) kits (Arbor Assays kit corticosterone #K014 and cortisol #K003, Ann Arbor, MI, USA) were used to quantify immunoreactive corticosterone and cortisol metabolites in SRW fecal solid-phase extracts (SPE). Two glucocorticoid assays were tested for two reasons: first, the dominant circulating glucocorticoid in SRW remains to be determined (no baseline plasma samples are available from unstressed animals, and both GCs are detectable in various tissue types); second, mammalian fecal metabolites of any GC commonly include an array of several metabolites, all of which can have varying affinities to immunoassay antibodies; thus, several antibodies are often compared, with the "best" antibody considered to be the one that detects highest concentration while also passing validations.

We conducted tests of parallelism and accuracy using standard methods described in Grotjan and Keel (1996). To test for parallelism, a pooled SRW fecal SPE extract was serially diluted in assay buffer to produce eight dilutions (range 1:1–1:128) which were then assayed as unknowns in both the cortisol and corticosterone EIAs, following which the slope of percentage of bound antibody vs. relative dose was compared to the slope of the known-concentration standards. Parallelism of the two binding curves (serially diluted pool and hormone standards) indicates that the antibody binds well to an immunoreactive component in the sample of interest, with very similar affinity as to pure parent hormone; this is considered strong evidence that the

hormone is in fact present in the sample (Grotjan and Keel 1996). Assay accuracy (aka "matrix effect test", "interference test") was next assessed by spiking a full standard curve with pooled 1:5 SRW fecal extract and assaying alongside a second standard curve that was spiked only with assay buffer. Accuracy was initially tested with a simple methanol extraction and then after performing SPE (see above: Steroid hormone extraction). The resulting graph of apparent total hormone concentration vs. known standard concentration was assessed for linearity and slope; a slope within the range of 0.7–1.3 (ideal slope = 1.0) indicates the assay correctly discriminates low-dose from high-dose samples without interference from sample matrix (i.e., fecal sample components; Grotjan and Keel 1996).

Following successful validations, samples were assayed at 1:5 for both hormones. Assays followed standard QA/QC criteria including a full standard curve, non-specific binding (NSB) and zero doses ("blank"), and a known-concentration control (i.e., 1000 pg ml^{-1} of hormone) in every EIA microplate, with assay of all NSBs, zeros, standards, unknowns, and controls in duplicate. Any sample that exceeded 10% coefficient of variation between duplicates was reanalyzed. Subsequent dilutions were performed if sample concentration exceeded the range of the standard curve. Intra-assay and inter-assay variations for all assays were < 10%. For antibody cross-reactivities, assay sensitivities, and other methodological details, see Hunt et al. (2017).

Statistical analysis

Parallelism results for the two GCs are plotted as the percentage of antibody bound vs. log [concentration]. An F test was employed to assess differences between the linear portion of slopes of the resulting binding curve for serially diluted SRW fecal pool and each assay's standard curve. Accuracy results were plotted as apparent total concentration (i.e., standard + SRW fecal pool) vs. known standard concentration and assessed by linear regression, with acceptable accuracy defined as $r^2 \geq 0.99$ and slope within 0.7–1.3. *F* tests and linear regressions used two-tailed tests with Prism 7.0c for Macintosh.

Based on DA determination, we classified the whales as "exposed to DA", when DA was within detectable levels by the LC–MS/MS method in the fecal sample, and "non-detectable DA", when the DA determination fell below the limits of detection. Descriptive statistics (means and coefficients of variation within groups and standard errors) were calculated in R (R Core Team version 3.4.2, 2017). We performed a Shapiro–Wilk test for normality in those groups that had a sample size greater than 10 (i.e., non-detectable DA, and live with non-detectable DA groups). Normality could not be assessed in "positive exposed to DA" group and the "deceased animals" group, due to low sample size of $n = 3$. Fecal glucocorticoid content is presented per gram of dried feces; to allow comparison to prior published literature on undried feces, we also present conversions to estimated hormone content of undried (wet) feces, assuming an average water content in right whale feces of 84%. Glucocorticoid content of whale feces is thought to be stable after death (Rolland et al. 2017); to verify this for SRW, we compared fGCm content of samples from live vs. dead animals with a Welch *T* test and with an alpha of 0.05. No significant differences were found, following which samples from live and dead animals were combined for further analyses. Differences between the "exposed to DA" group and the "not detectable DA" group were tested with a one tailed Welch *T* test. Potential effects of age class or reproductive state were examined only for the "lactating females" group. Due to small sample sizes, we could not assess normal ranges of fGCm for other age classes and life history stages. Statistical analyses were performed in R (R Core Team version 3.4.2, 2017). Results for hormone content are expressed as means ± standard error.

Results

Hormone assay validations

The accuracy test with a simple methanol extraction initially failed the validations (i.e., slope outside the desired range of 0.7–1.3). However, after performing SPE extractions, the accuracy test was acceptable for both assays of fGCm extracts, as indicated by a linear relationship between the observed and expected hormone concentration ($r^2 \geq 0.98$) and a slope within the desired range of 0.7–1.3 (corticosterone slope = 1.077; cortisol slope = 1.009; Fig. 2, bottom panels; C and D). Serially diluted SRW fecal SPE extractions yielded displacement curves parallel to the respective standard curves, with no significant differences in slope for either the corticosterone ($F_{1,9} = 0.42$; $P = 0.53$) or cortisol ($F_{1,9} = 0.51$; $P = 0.49$) assays (Fig. 2, top panels; A and B).

Domoic acid and fecal glucocorticoid metabolite levels

Three fecal samples from SRWs were positive for DA with levels ranging from 0.30 to 710 ± 75 µg DA g^{-1} dry weight (approximately equivalent to 0.05–113.6 ± 12 µg DA g^{-1} wet weight) (Table 1; for more details, see D'Agostino et al. 2017). The highest DA level was recorded for a lactating female observed next to her calf at the time of sample collection (BFA9, 710 ± 75 µg DA g^{-1} dry weight (approximately equivalent to 113.6 ± 12 µg DA g^{-1} wet weight)). The lowest DA level was detected in an adult whose sex could not be determined (BFA13, 0.30 µg DA g^{-1} dry weight

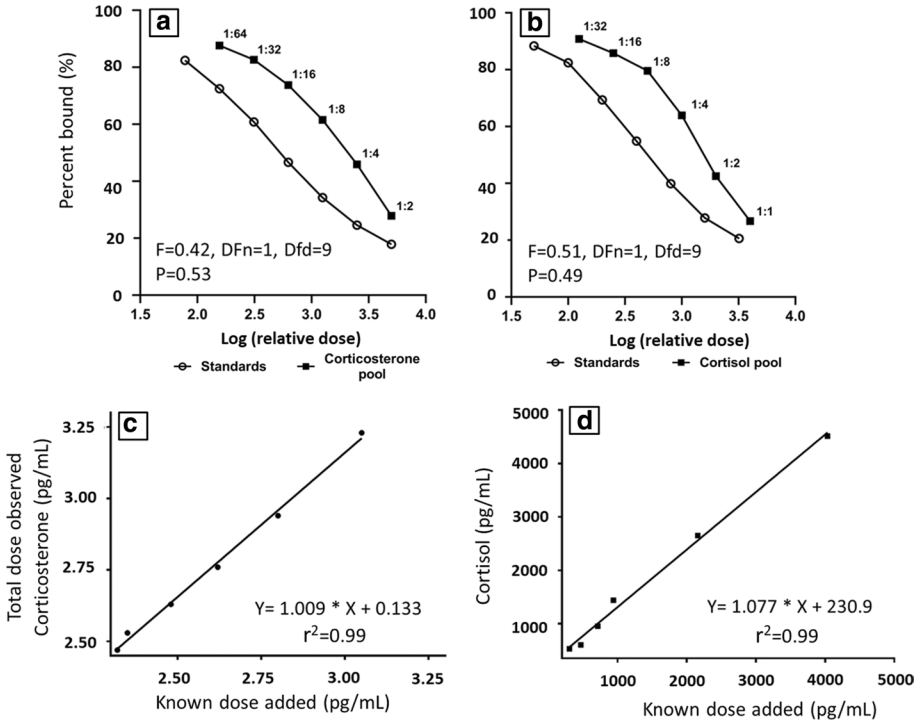

Fig. 2 Parallelism and accuracy results for corticosterone (**a** and **c**), and cortisol (**b** and **d**) enzyme immunoassays tested with pooled southern right whale (SRW) methanol fGCm extract. Parallelism (top **a** and **b**) was tested with serial dilutions of a SRW fecal pool SPE extraction, and the statistical results from F test slope comparison are shown in lower left. Accuracy (bottom **c** and **d**) was tested with 1:5 SPE extract; the best-fit regression equation is shown

(approximately equivalent to 0.05 µg DA g^{-1} wet weight)) (Table 1). Excluding the individuals who tested positive for DA exposure, we did not observe significant differences between the average fGCm quantified in samples collected from dead versus live whales (Welch *T* test $P = 0.25$ and $P = 0.38$ for fecal corticosterone and cortisol metabolites respectively; Online Resource Fig. S1). In whales with confirmed exposure to DA, both fGCm were lower in comparison to whales with undetectable fecal DA (Welch *T* test $P = 0.012$ and $P = 0.024$ for corticosterone and cortisol respectively; Fig. 3). Exclusion of samples from dead whales did not change direction or significance of these results (Welch *T* test $P = 0.015$ and $P = 0.032$ for corticosterone and cortisol, respectively; Online Resource Fig. S2).

Fecal GC metabolites and life history stage

Mean fGCm for presumed-healthy lactating females with no apparent exposure to DA (i.e., excluding the single lactating female with detectable DA) was 157.86 ± 50.13 ng g^{-1} and 65.99 ± 28.14 ng g^{-1} for fecal corticosterone and cortisol metabolites, respectively ($n = 7$). The highest fGCm level was observed in a lactating female (sample collected on 22-Dec-2018, BFA20; corticosterone fecal metabolites = 360.95 ng g^{-1}; cortisol fecal metabolites = 198.29 ng g^{-1}) in which DA was undetectable. Interestingly, at the time of sample collection, this female was observed with two calves and no other adults were sighted in the vicinity. Local whale-watching captains and guides

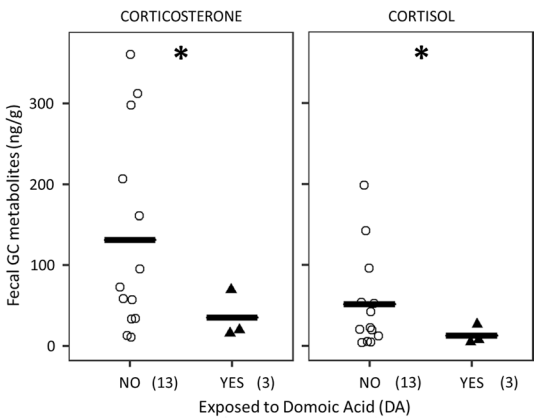

Fig. 3 Fecal glucocorticoid metabolite levels in southern right whales with (YES) and without (NO) detectable fecal DA, with immunoreactive fecal corticosterone metabolites shown at left and immunoreactive fecal cortisol metabolites at the right. Asterisks denote significant differences between groups, Welch *T* test $P = 0.012$ and $P = 0.024$ for corticosterone and cortisol, respectively. The black solid line indicates the mean for each group, and in parenthesis is the sample size for each group

reported seeing this individual with two calves in previous days (Table 1 and Online Resource Fig. S3).

On the other hand, both calves sampled in this study (BFA17 and BFA19) had similar levels of corticosterone fecal metabolites (33.26 ± 0.32 ng g^{-1}); however, the calf BFA19 had higher levels of fecal cortisol metabolites compared to BFA17 (Table 1). In addition, high fGCm content

were detected in an adult whale of unknown sex, BFA16, (312.38 ng g^{-1} and 52.35 ng g^{-1} for fecal corticosterone and cortisol metabolites, respectively; Table 1).

For 15 of 16 SRW fecal samples (excluding only one calf sample, BFA19), the corticosterone assay detected higher levels of fGCm than did the cortisol assay. Including all whales in this study, fCGm levels ranged from 10.98 to 360.95 ng g^{-1} for immunoreactive corticosterone fecal metabolites and 3.94 to 198.29 ng g^{-1} for immunoreactive cortisol fecal metabolites, with a mean of 113.54 ± 29.44 ng g^{-1} and 44.49 ± 13.96 ng g^{-1}, respectively (Table 1).

Discussion

To our knowledge, this study provides the first quantification of fGCm levels in whales exposed to DA. Although the sample size of this study is small, we observed significantly lower fGCm in samples from whales exposed to DA. These results are comparable to findings in California sea lions (*Zalophus californianus*) exposed to DA, where DA was associated with significantly reduced serum cortisol, tentatively attributed to abnormal function of the HPA axis. In the sea lion study, animals exposed to DA lacked the normal correlation between serum cortisol and pituitary adrenocorticotropin (ACTH), suggesting altered functioning of the HPA axis by DA (Gulland et al. 2012). Ultimately, the decreased fGCm associated with DA exposure might negatively impact the ability of exposed individuals to regulate metabolism and to cope with stressors.

Fecal samples collected from SRWs at their calving location in Península Valdés provide a unique opportunity to assess individual physiology. Obtaining fecal samples from SRWs throughout the year is difficult given the inaccessibility of their feeding grounds, mainly located in Sub-Antarctic waters (Rowntree et al. 2008; Valenzuela et al. 2009) and the Deep Ocean Basin and shelf break along the coast of Argentina (Zerbini et al. 2018). For this reason, the seasonal occurrence of whales in the gulfs that surround Península Valdés offers an exceptional opportunity to investigate in detail a part of their annual cycle, the period associated with mating, calving, and breeding. Notwithstanding, and although feasible, collecting fecal samples from whales at their calving grounds is not a simple task and requires significant survey efforts at sea. Moreover, in Península Valdés, SRWs feed only opportunistically, mainly during the spring when dense patches of mesozooplankton are composed of high-calorie prey such as calanoid copepods (Hoffmeyer et al. 2010; D'Agostino et al. 2016, 2018). Therefore, obtaining feces in the calving grounds is complicated by infrequent defecation as compared to elimination rates when whales are sampled in the areas primarily dedicated to feeding.

In line with other studies quantifying fGCm in whales (Hunt et al. 2006) and other mammals (Wasser et al. 2000), our corticosterone antibody detected higher levels of fGCm in SRW than did the cortisol antibody. However, this does not necessarily indicate that pure corticosterone is present in SRW feces, or that corticosterone is the primary circulating GC in plasma, since corticosterone antibodies often detect mammalian fecal metabolites of cortisol. For example, a corticosterone radioimmunoassay (RIA) has been recommended for quantification of fGCs in several mammalian species in which cortisol is known to be the major circulating GC in plasma (Wasser et al. 2000). High-performance liquid chromatography (HPLC) analysis of fecal extracts of the closely related NARW revealed that NARW feces contain at least nine separate glucocorticoid metabolites, none of which were pure cortisol or pure corticosterone. Nonetheless, a corticosterone assay detected most of these NARW fGCm, generating data that correlated well with the presumed stress status of the individuals and allowed identification of those impacted by chronic stress due to entanglement or vessel-related noise disturbance (Hunt et al. 2006; Rolland et al. 2012, 2017).

Glucocorticoids in both plasma and feces often vary significantly between different age classes, sexes and reproductive states (Keay et al. 2006). Hence, fGCm are useful biomarkers for studying the individual and population status (physiology, behavior) of free-ranging whales. Moreover, quantification of fGCm can discriminate between different ages, sexes, and reproductive stages in baleen whales (Hunt et al. 2006, 2019; Corkeron et al. 2017; Valenzuela-Molina et al. 2018). In NARW, pregnant and lactating females and mature males have higher fGCm than other demographic groups (non-reproductively active females, immature females, and immature males) (Hunt et al. 2006; Corkeron et al. 2017). Due to the small sample size, in this study we could not determine normal ranges of fGCm for most demographic groups, but we were able to perform a preliminary assessment of the typical range of fGCm levels in presumed-healthy lactating females. Lactation is commonly considered the single most energetically demanding life history stage in a female mammal (Kenagy et al. 1990), and in most mammals, lactation is known to entail elevated plasma GCs (Lightman 1992). Several studies have found elevated GCs in blubber, baleen or fecal samples of lactating female whales (Hunt et al. 2006; Corkeron et al. 2017; Valenzuela-Molina et al. 2018). In fact, in the present study, the highest fGCm levels were registered in a lactating female that was sighted with two calves (BFA20; Online Resource Fig. S3). Right whales typically give birth to a single calf every 3–5 years after a 12- to 13-month gestation period (Best 1994; Kraus and Hatch 2001), and observations of twin

births in right whales (i.e., associated with genetic evidence) are unknown (Best et al. 2015). In the present study, both calves with BFA20 showed normal behaviors and appeared to be in good condition (Best et al. 2015). However, we perceived that the female tried to avoid the larger calf (Calf 2 in Online Resource Fig. S3). An instance of genetically confirmed calf swapping has been documented in NARW, in which two mothers exchanged calves shortly after birth and then each nursed the "wrong" calf for months afterward (Frasier et al. 2010). Thus, it is possible for a right whale calf to follow the wrong mother, and for a right whale mother to nurse a calf that is not her own (Frasier et al. 2010). Irrespective of whether this female was the mother of both calves, or if both calves were nursing, it is likely that the resulting energetic burden and physiological stress could have resulted in elevated fGCm levels in this female.

Due to low sample size, we cannot fully separate the effect of DA from the possible effect of lactation. Most of our non-DA-detected samples were from lactating females, and since lactating females tend to have higher fGCm, the higher fGCm noted in the non-DA-detected group may be partially due to the confounding effect of lactation. In this study, low *n* precluded use of statistical models that could separate the effects of DA exposure and lactation. However, the scale of the difference in fGCm noted here exceeds the typical effect of lactation on fGCm in large whales. For example, in NARWs, lactating females have fGCm levels that are only mildly elevated compared to non-lactating females, similar to fGCm of immature males, and lower than fGCm of pregnant females and mature males. Thus, we suggest that the large difference in fGCm noted here between the DA and non-DA-detected groups is most likely attributable to the effect of DA exposure rather than lactation. Similarly, due to our inevitable low sample size, we could not separate the effect of potential environmental or oceanographic conditions prevailing in the different years or compare the effects of years considered of high (2003, 2005, 2007–2013), versus low calf mortality (2004, 2006, 2014–2019) (Marón et al. 2021). Greater sample sizes will be necessary to conclusively answer these questions, and we encourage cetacean fecal hormone researchers to include toxicological analyses where possible, and to archive samples of feces for future analyses.

Corticosterone levels of whale BFA16 (an adult of unknown sex) were the second-highest detected in this study. This sample was collected in proximity to a mating group, and thus we assumed that these feces were produced by an individual associated with the courtship group. Right whales have a promiscuous, scramble-competition mating system involving a single adult female surrounded by multiple males that actively compete for positioning near the female (Wells et al. 1999), an activity presumed to be energetically costly and hence stressful from a physiological perspective. In many mammals, individuals actively involved in reproductive courtship or competition have elevated fGCm. Mature male NARW sampled during the breeding season have elevated fGCm (Hunt et al. 2006); further, on a population-wide basis, fGCm tend to elevate in NARW from August to September as surface-active mating groups become more common (Rolland et al. 2012). In some cases, immature individuals are also observed in these groups (Kenney 2009). Therefore, the high levels of corticosterone fGCm detected in this whale may have been related to the physiological stress associated with mating activities.

The fecal samples collected from the two live calves had similar fecal corticosterone metabolite levels and calf BFA19 was the only whale whose fecal cortisol metabolite levels were higher than the corticosterone metabolites. Several studies have suggested that the GC levels in baleen whale calves are highly variable because they may ingest maternal hormones through milk and then concentrate them in their feces (Hunt et al. 2006, 2019; Fernández Ajó et al. 2018); in this study, both calf samples were roughly in the middle of the range seen in other individuals, but more calf samples will be necessary to determine how fGCm levels of nursing calves compare to those of non-nursing animals.

In the present study, three fecal samples of deceased individuals were recovered at necropsy from stranded SRW, one from a juvenile male and the other two from unknown sex individuals. Whale BFA6 was a juvenile male necropsied by the Southern Right Whale Health Monitoring Program on October 5th, 2014 (100514PV-Ea18; for details see Alzugaray et al. 2020). Unfortunately, no data associated with the dead whales in 2013 (BFA1 and BFA2) such as sex, age class, photographs or health condition were registered. Therefore, we cannot know if the fGCm values recorded in these whales might be attributable to exposure to stressors experienced before death. In NARW, fGCm levels from samples collected at necropsy correlate with cause of death, with entanglement in fishing gear associated with higher fGCm than sudden death due to vessel strike. In extreme chronic stress (e.g., illness, anthropogenic disturbances, exposure to phycotoxins during HABs), the HPA axis can begin to fail, with circulating GC levels eventually declining below normal. Consequently, physiological state may be altered, immune system function depressed, normal activities such as feeding, reproduction, lactation disrupted and, thus, the survival of the individual is reduced (Dickens and Romero 2013; Rolland et al. 2017). Several experiments in stress physiology, both in the laboratory and the field, have reported that declines in GCs often occur under prolonged or repeated exposure to chronic stressors (Rich and Romero 2005; Dickens and Romero 2013). In SRW calves and in humpback whales (*Megaptera novaeangliae*) exposed to chronic stress, baleen GC content rises gradually for weeks as health declines, but then falls sharply just prior to death

as individuals become moribund (Fernández Ajó et al. 2018; Gabriele et al. 2020). The three cases presented here will hopefully be the start of a growing dataset to which other researchers can add, ultimately enabling future analyses that can further investigate whether fGCm in samples collected from necropsies of SRWs can illuminate cause of death.

Conclusions

Our results provide the first evidence that HAB-associated neurotoxins such as DA may affect adrenal physiology in whales. In accordance with the results reported for sea lions (Gulland et al. 2012), we observed a decline in fGCm correlating with DA exposure. If ingestion of phycotoxins can result in long-term suppression of baseline GCs, or of the ability of the HPA axis to respond to stressors, marine mammals could suffer reduced ability to cope with subsequent stressors such as predator attacks, pathogens, environmental changes, or anthropogenic factors, among others. Adrenal function is essential to maintain circulating blood glucose and other aspects of metabolism within normal bounds, while the ability to elevate GCs facilitates energy mobilization to physiologically cope with the stressful event and to initiate appropriate behavioral responses. Various toxicants have been shown to reduce adrenal function across taxa (Romero and Wingfield 2016) and could have negative consequences on the ability of cetaceans to respond and adapt to ongoing environmental and anthropogenic changes.

Future directions

Traditional endocrinological methods of analysis include blood sampling from individuals, but this is not possible for large whales (Hunt et al. 2013). Therefore, sampling and analysis of non-traditional matrices such as feces, respiratory vapor, and blubber in combination with collection of samples of baleen, earplugs, and feces from dead individuals would likely increase sample sizes and thus our understanding of the interrelationships among DA exposure and age, sex, and reproductive status of cetaceans. Given that chronic exposure to DA could alter the HPA axis as well as the hypothalamus–pituitary–thyroid axis (Arufe et al. 1995; Alfonso et al. 2000), we suggest that conservation physiology studies in marine mammals exposed to phycotoxins should incorporate analysis of other adrenal and thyroid hormones. For example, the reproductive hormones progesterone and testosterone metabolites could be used to infer reproductive state, and thyroid hormone metabolites could aid in assessing the nutritional and metabolic status and its correlation with exposure to toxicants. Based on our results and those of Gulland et al. (2012), as well as several studies indicating that HABs are becoming more frequent and intense worldwide (Van Dolah 2000; Masó et al. 2006; Erdner et al. 2008), we emphasize that monitoring programs aimed to evaluate the health status of marine mammal populations should include the collection of samples that allow investigation of stress physiology for understanding the impacts of natural and anthropogenic stressors on marine wildlife.

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Author contribution statement VCD originally formulated the idea. VCD, MD and MMU did the fieldwork. VCD and AFA conducted sample extraction and data analyses. BK, KEH and CLB helped with analytical data in the laboratory. BK provided the funding for domoic acid extraction and MMU for dead whale sampling. AFA performed the fGCm validations, fGCm assays, and the statistical analyses. VCD and AFA wrote the manuscript. All authors contributed critically to the manuscript and gave final approval for publication.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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