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

--Manuscript Draft--

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Response to Reviewers:	Comments to Authors from Handling Editor Thank you for submitting to Oecologia. We don't see a ton of whale papers, so this is exciting. As you will see, the reviewers liked the paper overall. They each had suggestions for improving the manuscript. The most common theme was that the authors recognized the small sample size was a problem (but it is a study on Southern Right Whales, not zebrafish!), and they acknowledged it. However, it may need to be acknowledged up front, as soon as the abstract. I don't disagree with this. However, the work is broadly interesting enough to warrant publication after revisions. The other largest request was to perhaps downplay the method validation as a main thrust of the paper. Yes, the validation is important, but is that the focus of the paper, or is it the broader biology and potential problems of DA? Two reviewers thought a little more focus on the biology and a little less on the method validation was warranted. I have to agree since Oecologia has a broad readership. Overall, the suggested changes can be made in a careful revision. Reply: We greatly appreciate the Handling Editor's comments about our study. Taking these suggestions into account, we have carefully and thoroughly revised our manuscript, including adding a mention of the small sample size to the Abstract, and, in the main text, reducing emphasis on validations in favour of more emphasis on the broader biological questions. We have also either reformulated some sentences or included additional references in order to comply with some specific comments. Reply to Reviewer # 1 Reviewer #1: Overall I found very little in this manuscript for which I would suggest edits or revisions, in fact this is one of the best-written manuscripts I have reviewed. I do have a few very minor comments that may or may not need to be addressed with revisions, but that might strengthen the paper somewhat or provide additional clarification. The use of corticosteroids as a HAB-related biomarker is very useful and the description of this method will be of significant importance to researchers doing similar work. The authors did an excellent job of laying out the background mechanisms of DA production, toxicity and endocrine pathways that can be altered during acute and chronic DA-related stress. The background section was exceptionally well-written and useful to provide all necessary relevant literature and clearly describe the research problem being addressed. The Study Species paragraph in the Methods section seems to be background information, and might be more appropriate to include in the Introduction section instead. Reply: We greatly appreciate Reviewer # 1's comments. We tested moving the

paragraph about SRW natural history to the Introduction as suggested but found that it disrupted the Introduction's focus and flow; we feel that thought the natural history of the study species should be understood by the reader, sometimes a paper flows better if such material is presented in Methods in a paragraph about the study species. Ultimately, we have moved this paragraph back to Methods, but we do appreciate the suggestion.
Reviewer #1: It appears that a Welch's unequal variances t-test was used to compare the concentrations of fGCm between DA-positive and DA-negative groups, but this test assumes normal distributions for both groups. It was unclear if this assumption was true in the case of the DA-positive vs. DA-negative groups, but should be more clearly stated in order to say there was a significant difference between the two, since the DA-positive group only included 3 samples. However, this may be a moot point since the authors suggest but do not conclude that DA is responsible for changes in fGCm, but may also be confounded by other life history factors.
Reply: We agree with Reviewer #1's observation regarding the assumption of normality needed for performing a Welch's t-test, complied with this, in lines 285-288 of the revised manuscript we stated: "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."
Reviewer #1: line 447 - "Our results provide the first evidence that HAB-associated neurotoxins such as DA can affect adrenal physiology in whales" - since the authors state that DA *might* be a factor in changes to fGCm levels, I would suggest wording that reflects this, so the reader does not think that the authors are making a conclusion that DA *does* affect fGCm levels. Perhaps changing the sentence to read "...first evidence that HAB-associated neurotoxins such as DA may be a factor affecting adrenal physiology in whales"
Reply: We agree with this suggestion and have reformulated the sentence as follows: "Our results provide the first evidence that HAB-associated neurotoxins such as DA may affect adrenal physiology in whales." Please see lines 479-480 in our revised manuscript.
Reviewer #1: Table 1 - the authors present DA concentrations ranging between 0.310 and 710 ug/g. It would be useful to provide a bit of reference data for other marine mammal DA values from the literature for comparison. Mainly because the highest reported DA concentration from a marine mammal that I am aware of is from the 2010 Fire et al. paper in Aquatic Mammals, where they reported DA in feces at 258.67 ug/g from a whale stranding during a California mass mortality event co-occurring with a severe Pseudo-nitzschia bloom. The maximum DA value from this manuscript is nearly 3 times greater, but from an individual likely only opportunistically feeding, and in a region not as well-known for severe P-n blooms. I would be interested in hearing the authors thoughts on possible explanations for this, as this is a very exciting finding. Also, this extremely high DA value should be emphasized as a major finding of the paper, and I would suggest including this in the abstract and the discussion.
Well done!
Reply: We greatly appreciate this suggestion and comments about our findings; however, as we explained in our manuscript, the data on positive DA levels in southern right whales were previously published in D'Agostino et al. (2017) (please see lines 139-141. In the present study and in D'Agostino et al. (2017) the DA levels in fecal samples were presented in µg/g of dry weight (by lyophilization), which will result in different levels when compared to DA in non-lyophilized fecal samples. Nearly all published studies for DA in marine mammal fecal samples have used non-lyophilized samples (and thus expressed in wet weight). Therefore, in order to make our data comparable to previous DA-related marine mammal studies, in our previous study, D'Agostino et al. (2017), we have determined the mean water content in fecal whale samples from southern right whales (84%). Therefore, although our DA values were high, considering the water content in fecal samples, our measured values are not the highest reported for a marine mammal species. Nevertheless, our DA values quantified in southern right whales were higher than those reported in feces from North Atlantic right whales on their feeding grounds in the Great South Cannel and the Bay of Fundy (Leandro et al. 2010; and for more details see D'Agostino et al. 2017). In addition, the highest levels of DA detected in our study were the highest registered in a living right whale (710 µg DA g-1 dry weight or 113.6 µg g-1 wet weight assuming a mean water content of 84%); thus, it can be deduced that this whale was exposed to an intense toxic Pseudo-nitzschia bloom at the breeding ground of Península Valdés and these

data were discussed in D'Agostino et al. (2017). However, following the reviewer's suggestion and for the sake of clarity, we have included information about this as follows: "Three fecal samples from SRWs were positive for DA with levels ranging from 0.30–710 ± 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)." (Please see lines 313-315 and throughout our revised manuscript).

D'Agostino VC, Degrati M, Sastre V, Santinelli N, Krock B, Krohn T, Dans SL, Hoffmeyer MS (2017) Domoic acid in a marine pelagic food web: Exposure of southern right whales Eubalaena australis to domoic acid on the Península Valdés calving ground, Argentina. Harmful Algae 68:248-257 https://doi.org/10.1016/j.hal.2017.09.001

Leandro LF, Rolland RM, Roth PB, Lundholm N, Wang Z, Doucette GJ (2010) Exposure of the North Atlantic right whale Eubalaena glacialis to the marine algal biotoxin, domoic acid. Marine Ecology Progress Series 398:287–303.

Reply to Reviewer # 2

Reviewer #2: The authors investigated whether domoic acid (a toxin produced by diatoms) correlated with faecal GC metabolites in southern right whales. In addition, they validated the EIAs for measuring faecal cortisol and corticosterone for this species. Whales with evidence of domoic acid exposure had lower fGCm levels than whales with undetectable domoic acid levels. The authors also report that the highest fGCm level was found in a lactating female without detectable domoic acid levels. The authors acknowledge that their sample sizes were low, but that their study provides preliminary evidence that exposure to domoic acid could alter adrenal function.

The immense effort required to collect this data should be appreciated. I think it can be easy for some readers to be critical of the sample size; however, accessing this type of physiological data from a large, highly mobile, aquatic mammal is phenomenal! The authors have clearly stated the caveats and limitations of the data and have stated that the results are best-suited to serving as a roadmap for future research.

Overall, the manuscript is very well-written. It is clearly and logically set up, and the methodology is detailed. The authors were very careful in using best-practices when performing their hormone metabolite validations and measurements, and this is evident in the methods.

I have a few comments, along with some other minor suggestions, that I hope will be useful.

Reply: We greatly appreciate Reviewer #2's comments and observations about our paper. Taking Reviewer #2's suggestions into account, we have attentively revised our manuscript and have reformulated the text accordingly (please see below).

Reviewer # 2's comment 1: Cohesiveness of objectives and scope: I think there is an opportunity to focus the objectives of the study and increase the fit of the manuscript with a broad ecological journal (as opposed to a journal more focused on physiology, for example). My suggestion would be to set up the objectives to put the focus of the work on (2) and (3) (i.e., state the objectives related to DA as the main goals). This could be followed with a statement that, to accomplish these goals, an enzyme immunoassay was validated to quantify fGCm in faeces of SRW for the first time. Further, in relation to keeping the goals consistent and in-line with title of the paper, quantifying fGCs in lactating females feels a little bit out of place to me as well. However, I agree the data are valuable and should be reported here, I just might consider leaving this out as a core objective of the study unless there is a way to tie it more clearly to the work linking hormones and DA. Finally, I think the paper would benefit from the inclusion of a prediction for how fGCm levels will relate to DA. Although these would be minor changes to the text of the paper, I think it will align the paper more closely with the scope of Oecologia, while also allowing others who are interested in the finer details of the hormone validations to still access that information easily.

Reply: We agree with the reviewer's comment and have revised the objectives to place the focus of our research on the broader biology and potential problems of DA exposure of relevance for Oecologia readership as follows: "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.", see lines 136-139 of the revised manuscript. We have also excluded the assessment of typical fGCm for lactating females from the main objectives, but we do include it in the results as we consider it valuable data to report here and it aids with data interpretation. Lastly, within lines 76-86 we have included

predictions regarding the adrenal response to exposure to DA in the revised manuscript as follows: “As an additional complication, domoic acid is an excitatory amino acid analogue 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 Mahes 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 physiological impacts of exposure to DA.”

Reviewer # 2’s comment 2: I think it would be useful to provide an estimate of the timeframe faeces will reflect DA levels and fGCs (even if this is an approximation based on other species). This will allow readers to understand what a faecal sample collected from a live or dead animal is reflecting - i.e., does it correspond to a very recent exposure to DA and over what total time period is it likely integrating GC levels.

Reply: Although clearance studies have not been conducted on southern right whales, several studies have documented that DA is cleared rapidly in mammals (Iverson et al. 1989; Truelove and Iverson, 1994; Maucher and Ramsdell 2007). For example, in sea lions it has been estimated that clearance occurs within 48 h of ingestion (Wittmaack et al. 2015). These findings suggest that the southern right whale individuals analysed in our study were exposed to DA recently—likely within a few days. Concerning the fGCm, clearance studies have not been conducted on southern right whales. 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. Therefore, following the reviewer’s suggestion, we have included information about this in our revised manuscript (Lines 173-184 and now reads as follows: “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 SRW’s analyzed in our study were exposed to DA recently and the fGCm can be correlated with the DA exposure.”

Iverson F, Truelove J, Nera E, Tryphonas L, Campbell J, Lok E (1989). Domoic acid poisoning and mussel-associated intoxication: preliminary investigations into the response of mice and rats to toxic mussel extract. Food and Chemical Toxicology 27(6): 377–384.

Maucher JM, Ramsdell JS (2007) Maternal-fetal transfer of domoic acid in rats at two gestational time points. Environ. Health Perspect. 115:1743-1746.

Millspaugh, JJ, Washburn BE (2004) Use of fecal glucocorticoid metabolite measures in conservation biology research: Considerations for application and interpretation. General and Comparative Endocrinology, 138(3), 189–199. https://doi.org/10.1016/j.ygcen.2004.07.002

Rolland, R. M., Parks, S. E., Hunt, K. E., Castellote, M., Corkeron, P. J., Nowacek, D. P., Wasser, S. K. & Kraus, S. D. (2012). Evidence that ship noise increases stress in right whales. Proceedings of the Royal Society B: Biological Sciences, 279(1737), 2363–2368. https://doi.org/10.1098/rspb.2011.2429

Rolland, R. M., Hunt, K. E., Doucette, G. J., Rickard, L. G. & Wasser, S. K. 2007 The inner whale: hormones, biotoxins and parasites. In The urban whale: North Atlan- tic right whales at the crossroads. (eds S. D. Kraus & R. M. Rolland), pp. 232–272. Cambridge, MA: Harvard University Press.

Truelove J and Iverson F (1994) Serum domoic acid clearance and clinical observations in the cynomolgus monkey and Sprague-Dawley rat following a single IV dose. Bulletin of Environmental Contamination and Toxicology, 52(4):479-486.

Wittmaack C, Lahvis GP, Keith EO, Self-Sullivan C (2015) Diagnosing domoic acid toxicosis in the California sea lion (Zalophus californianus) using behavioral criteria: A novel approach. Zoo biology, 34(4):314-320.

Reviewer # 2's comment 3: For the discussion, is there anything else about the three animals exposed to DA that could be leading to the lower GC levels besides lactation? For example, all the individuals were not all sampled at the same time of year and the samples are coming from a wide year range (2013-2018) - is there any other environmental stressor that could be responsible? It might be worthwhile to state that you do not expect that other environmental pressures differed (or discuss others that could have differed as alternative reasons for the results).

Reply: We greatly appreciate Reviewer #2's suggestion. To address this, we have included a line in the Materials and methods section to clarify that all samples were recovered during the SRW breeding season. It now reads as follows: "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)." (Please see lines 165-167. Additionally, we have included in the discussion more details about possible variables that might influence fGCm levels in different years but, given our inevitably low sample size, we could not address the potential effect any other environmental stressor in the different years of fecal sample collection. The new sentence now reads as follows: "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 nor compare the effects of years considered of high (2003, 2005, 2007–2013), versus low calf mortality (2004, 2006, 2014–2019) (Marón et al. 2021)." Please see lines 426-430

Reviewer # 2's comment 4: The authors suggest a number of important future research goals. Given the sample size, I think this is a really important goal of the overall paper, as stated in the introduction (i.e., that the data is a preliminary assessment to identify avenues for further research). As a result, I think the discussion should have a strong section on future work. The authors mention in the conclusion that other physiological traits could be measured. Perhaps this could be moved up to the main part of the discussion and there could be a bit more detail as to why those traits in particular could be useful (i.e., what extra information will be garnered from aldosterone, thyroid hormones, etc.) and whether any other traits related to other aspects of physiological function could be useful (e.g., metrics related to immune function or reproduction given the sublethal impacts outlined in the introduction). In addition, what is needed in terms of further research to allow these types of analyses to benefit the conservation of SRW (i.e., how can the preliminary results presented in the manuscript best be scaled up to give more conclusive evidence that DA is a threat to the species)?

Reply: We appreciated reviewer #2's suggestion and have thus included in our revised version of the manuscript a new section "Future Directions" in which we aim to address all the suggestions made by Reviewer #2's comment 4. The new section now reads as follows: "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." Please see the new section in lines 493-510.

Minor comments:

Line 84-85 - I am wondering if you could add an additional sentence about the mechanism behind lowered GCs in response to DA to give an indication of why the

HPA axis might be suppressed instead of activated? This would also lead to greater understanding by readers before providing a prediction for SRW.
Reply: We appreciate the reviewer's suggestion. In lines 76-86 we have stated that we could predict that fGCm would rise during DA exposure as a generalized response to DA-related illness. However, we also referenced a previous study (Gulland et al. 2012) which has reported the opposite correlation in sea lions exposed to DA, which could be explained if DA exposure lead to adrenal function insufficiency. We opened this paragraph also mentioning that DA is an excitatory amino acid analogue of glutamate, which in turn is an excitatory amino acid that plays an important role in the activation of the HPA axis, regulating many pituitary hormones involved in the stress response. We think that the information provided in these lines is based on the best current knowledge. When exploring this correlation we could not predict a directionality in the regulation of the HPA axis, but we considered the two alternatives as possible outcomes.
Line 162 - Can you please add the range of months in which the samples were collected.
Reply: Taking reviewer #2's suggestion into account, we modified the sentence as follows: "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)." Please see lines 165-167 of the revised manuscript.
Line 270 - Whales were divided into categories of either a detectable or undetectable level of DA. Perhaps this could be stated above in the domoic acid methods section as I had to scroll back up to make sure I understood how this was quantified. For example, at Line 192 you could indicate that DA was measured as ug/g, but whales were classified as having either detectable or non-detectable levels.
Reply: Addressing reviewer #2's suggestion, we have made the following clarification in the Materials and methods section lines 281-283 "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."
Line 417 - "GCs" should be "GC"
Reply: Complied with. We greatly appreciate Reviewer #2's observation. Please see line 449 of the revised manuscript.
Line 428 - add the word "or"
Reply: Complied with. The sentence has been corrected as indicated by Reviewer # 2. Please see line 460 of the revised manuscript.
Line 436 - "individuals" should be "individual"
Reply: Complied with. "individuals" has been replaced by "individual". Please see line 468 of the revised manuscript.
Table 1 - This table may be easier to read if there is space to use full label headings of corticosterone and cortisol metabolites instead of B and F.
Reply: Taking reviewer # 2's suggestion into account, we have reformulated table 1 accordingly. Please see Table 1 in our revised manuscript.
Figure 2 - I suggest larger axis labels and legends to make the graphs easier to read.
Figure 3 - Open circles (rather than solid black dots) might display the data more clearly here.
Reply: Taking reviewer # 2's suggestions into account, we have reformulated figures 2 and 3, and the supplementary material accordingly. Please see Figures 1 and 3, and the supplementary figures Fig.S1 and Fig.S2 in our revised manuscript.

Reply to Reviewer # 3
Reviewer #3: General Comments
The authors present a well-written and methodologically thorough study examining the levels of fecal glucocorticoid metabolites (fGCm) in the Southern Right Whale (SRW) and the potential relationship of fGCm levels with exposure to the HAB-produced neurotoxin, domoic acid (DA).
Reply: We greatly appreciate Reviewer #3's comments and observations about our paper. We have attentively revised our manuscript and have reformulated the text taking Reviewer #3's suggestions into account and have replied to the specific comments and questions below.
The key statement in the Discussion section of the manuscript is found on lines 388-398: "Due to low sample size, we cannot fully separate the effect of DA from the possible effect of lactation." Hence, the authors have correctly indicated in their title the

"potential" endocrine correlation with DA exposure. I understand and appreciate the difficulty associated with obtaining these types of samples and thus the very small sample size. Accordingly, the authors have not overstated their conclusions, but have provided some very interesting and valuable data that can be built on by similar continuing efforts in the future. I might suggest that the authors refer briefly, but more specifically, to this potential caveat in the abstract.
Reply: We greatly appreciate that Reviewer #3 recognizes the difficulties associated with obtaining these types of samples. In lines 50-52 of the abstract, we now acknowledge this limitation as follows: “Though 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.”
In terms of methods, it would have been highly beneficial if the authors had conducted spike-recovery experiments in order to assess the efficiency of the extraction protocol employed here. As noted below in the Specific Comments, heat produced by the 15 min sonication step may have caused some degradation of the target hormones (I am unsure as to the temperature stability of fGCm); however, spike-recovery experiments would aid in determining if this is a potential problem.
Reply: We greatly appreciate Reviewer #3’s comments and suggestions. However, percentage-recovery of added radiolabeled hormone was not tested in this study, since this method of testing extraction efficiency is not applicable to solid-tissue sample types such as feces, shed skin, hair, baleen, etc. This is because the added radiolabelled hormone does not behave like native hormone, as Palme et al. state in their 2013 review. It is not possible to mix liquid parent hormone into a solid sample type in a way that adequately mimics the nature of deposition of native hormone into the gut and the fecal matrix. Native hormone and hormone metabolites are presumably bound into the solid particles of dried fecal sample; added radiolabelled hormone could only loosely adhere to the surface of the dried particles and thus would be more easily extracted. Further, for feces in particular, an additional, and major, issue is that fecal steroid hormone metabolites are not chemically identical to the parent hormone that circulates in plasma. For example, in the closely related North Atlantic right whale, HPLC analyses indicate that circulating cortisol is metabolized to a complex of at least nine different immunoreactive fGCms (Hunt et al. 2006) — none of which are commercially available in the radiolabelled form that would be necessary to do spike experiments. For these reasons, spike experiments to assess percentage recovery are generally not advised for mammalian fecal hormone studies (Palme 2019). It is for this reason that fecal endocrinology, as a field, tends to focus on relative patterns rather than absolute concentrations. The most relevant question, in our view, is: do the relative patterns of hormones (i.e., regardless of the % recovered) reflect the physiological state of the animal? If so, the assay has predictive and explanatory utility, regardless of % recovery. If not, it is not a useful method, regardless of % recovery. For this reason, our analysis focuses on relative patterns and not on absolute concentrations. We have included a sentence in the Materials and methods section clarifying that % recovery was not tested and adding a citation to reviews that justify this course of action. Please see lines 232-236 “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.”
Hunt KE, Rolland RM, Kraus SD, Wasser SK (2006) Analysis of fecal glucocorticoids in the North Atlantic Right Whale (Eubalaena glacialis). General and Comparative Endocrinology 148(2):260-272.
Palme R, Touma C, Arias N, Dominchin MF, Lepschy M (2013) Steroid extraction: get the best out of faecal samples. Wien Tierarztl Monatsschr 100(9-10):238-46
Palme R (2019) Non-invasive measurement of glucocorticoids: advances and problems. Physiology & behavior 1;199:229-43.
Another, potentially more important methods issue is that the authors' 'matrix effect test' did initially show a matrix effect, which was then corrected by employing SPE; however, it was not stated whether the pooled, serially diluted SRW fecal extract used in the 'parallelism' test (line 228) was also subjected to SPE clean-up. If so, there is not an issue, but if not, then the dilution is serving to dilute both the target analyte and the matrix, thereby complicating interpretation of the results. From their matrix test data, it seems that an extract dilution of 1:5 (or less) and perhaps more, does cause a matrix effect in the EIAs, thereby requiring SPE to eliminate this effect. Use of SPE in the

parallelism test should be clarified in the text. Overall, the authors have presented a solid and valuable piece of work, even given the fact that they had few samples to work with as a result of the immense challenges associated with obtaining fecal samples on the SRW calving grounds, where defecation is very limited. The data analysis is thorough and the discussion of the results is tempered appropriately, given the very small sample size. As noted above, the findings presented here should be valuable in informing future efforts to better understand the potential effects of domoic acid on the SRW population, which is extremely challenging to study in a non-invasive manner.
Reply: We greatly appreciate Reviewer #3's comments and observations about our paper. We have carefully revised our manuscript and have reformulated the text taking Reviewer #3's suggestions into account. Please see our responses to the specific comments and questions below for more details.
Specific Comments
Line 60: replace 'Broadwate' with 'Broadwater'; and correct reference to read: Broadwater MH, Van Dolah FM, Fire SE (2018) Vulnerabilities 542 of marine mammals to harmful algal blooms. Harmful Algal Blooms: A Compendium Desk Reference. John Wiley & Sons, Ltd. Pp. 191-222
Reply: Complied with. Following the reviewer's observation, we have amended this mistake in the revised version of our manuscript. Please see line 61 and references in our revised manuscript.
Line 175: it would be good to understand what comprises 'lysing matrix D', if not proprietary; at least some sense of what this matrix contains, if the exact formulation is not available
Reply: Taking Reviewer # 3's comment into account, we added a description of lysing matrix D and the sentence now reads: "…FastPrep tubes containing 0.9 g ceramic beads (lysing matrix D, Thermo Savant, Illkirch, France),…" Please see lines 189-190 in our revised manuscript.
Line 186: it is not apparent from the D'Agostino reference whether effects of the fecal matrix were taken into account for the calibration of the LC-MS/MS analysis; accuracy of DA quantification could definitely be affected by the fecal matrix - how did the authors address this uncertainty if potential (likely) matrix effects were not accounted for?
Reply: Matrix effects that possibly could influence DA values were not considered in this study. This decision was taken due to the low availability and high price of DA standards, but more importantly accurate DA determinations were outside the scope of this work as to date nothing is known about the relationship of DA levels in fecal pellets and the health status of marine mammals. In this study, the aim was to investigate a possible correlation between DA and hormone levels and a potential systematic error in DA values would not affect the results of the study.
Line 187: it is not clear why the authors chose to exhaustively extract the material 20 times - this seems quite excessive! Was there a rationale or precedent for this?
Reply: We do not have an explanation for the phenomenon of the retention of DA in lyophilised fecal samples. DA concentrations after repeated extraction steps were low, but still clearly detectable. Surprisingly after 11-20 extraction steps DA levels did not show a gradual decrease from one extraction step to the next. We also cannot say anything about recovery, because adding DA to fecal pellets under these circumstances would never result in representative recoveries, as it is well known that added analytes show far better extractabilities than analytes that have to be released from the sample matrix.
Line 191: the authors should provide some indication of what this 'correction factor' was, which appears to have been based on the results from extractions 3-20; the statement that these extractions contained only 'minor additional DA' (line 188) is not sufficiently clear to justify a 'correction factor' based on such results being applied to their analytical data.
Reply: The correction factor was calculated on based that of the total DA of the 20 extractions in the sample BFA9 (which presented high values of DA that fell outside the calibration range), the 38% were found in from 3rd to 20th extractions. Therefore, based on this finding, the two other samples (BFA11, BFA13) positives to DA were only extracted twice, with final DA content estimated as the sum of DA in the first extract and second extract, plus the correction factor of 0.38 based on data from the 3rd-20th extractions of sample BFA9. Considering the observations made by Reviewer # 3, we have now included information about the correction factor in our revised manuscript as follows: "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). In order 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." Please see lines 202-208 in the revised manuscript

Lines 191-192: the authors need to provide the limit of detection (LOD) and limit of quantification (LOQ) for their LC-MS/MS method

Reply: Complied with. We have specified the limit of detection (LOD) and limit of quantification (LOQ) for our LC-MS/MS method. Please see the caption of Table 1 in the revised version of our manuscript.

Line 214: sonication, especially probe sonication (although it is not clear whether probe or bath sonication was used here), can generate considerable heat; did the authors monitor temperature in this extract and/or attempt to control the temperature in order to avoid potential degradation of the target hormones?

Reply: Thanks for this observation. In this study we utilized a bath sonication. We have included details in the revised manuscript clarifying the type of sonication and equipment used as follow: "…bath sonicated for 15 min (Branson 3800 ultrasonic cleaner)…" Please see lines 230-231 Bath sonication for 15 min does not produce significant heat as tubes are submerged through the process in room temperature water (approximately 20°C). We have monitored the temperature inside a glass tube before and after 15 min bath sonication and have not perceived a significant change in the temperature (Please see photographs in the response letter).

Line 223: is it known whether other fecal metabolites unrelated to GCs may have epitopes recognized by antibodies employed in the two kits tested?

Reply: Thank you for your comment. We refer Reviewer 3 to the tables for #K014 corticosterone (top) and #K003 cortisol (bottom) kits cross reactivity reported by the manufacturer Ann Arbor, MI, USA. We believe that mentioning the specific kits used and manufacturer should provide enough information for the readers regarding the specificity and cross reactivity of the assays used in this study. (please see tables in the response letter)

Lines 278-280: it does appear that the diluted pool extract shows two distinct segments to the 'curve', with dilutions 1:1 through 1:4 being of higher slope than for dilutions 1:4 through 1:16. The latter looks (by eye) to be more in line with the standards curve - again, relating to my question above, were these dilutions of the pool extract subjected to SPE clean-up? This needs to be clarified. If SPE was not done, this could explain the better agreement in slope with dilutions greater than 1:5.

Reply: We much appreciate reviewer #3's comment. In compliance with the reviewer's comments and suggestions we have make amendments in the revised manuscript as described below.

In the parallelism test, an F statistic test is used 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. It now reads in the revised manuscript as follows: "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." (Please see lines 275-277 in the Materials and methods section).

With regards to the parallelism test performed with SPE, we appreciate the reviewer's observation and have addressed this issue. In the revised version, we reported the parallelism test performed after SPE, and we have made the necessary clarifications throughout the text specifying that SPE extractions where used to perform the validations. In the revised manuscript Materials and methods section's lines 239-240 we now state: "…corticosterone and cortisol metabolites in SRW fecal solid phase extracts (SPE)." whereas in the Results' section it now reads: "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)." please see lines 308-311 and also this was clarified in figure's #2 legend.

- Line 288: it is not clear whether the error term applies only to the 710 value (see line 291, where the same value is given for the error term with the 710 value; also, the same is true in Table 1)

Reply: Taking Reviewer # 3's comment into account, we have clarified this as follow: "Three fecal samples from SRWs were positive for DA with levels ranging from

	0.30–710 ± 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)." Please see lines 313-315 in the revised version of our manuscript. Our reply to Reviewer # 3's comment: this the only DA value of the three positive samples with an associated error term, presumably based on the three 'technical replicates' referred to on line 186? Why were two additional aliquots of the other positive samples not also extracted in order to provide comparable replication and error terms? Yes, Reviewer #3 is right, the DA level from BFA9 is the only DA value with an associated error term due the methodology applied for DA extraction from the fecal sample. In this study we did not aim to determine the absolute DA values in SRW fecal samples, but instead we aimed to determine if DA was detectable or not detectable and assess the correlation with fGCm. The absolute values in this context do not yield relevant information for the work; therefore, the effort and costs associated to generating them was deemed not justifiable. Figures Lines 817-821 (Fig. 2 legend): the individual graphs need to be labelled with A, B, C, or D. Both x- and y-axes on all graphs require the addition of titles and units. Reply: Thanks for this observation, we have addressed this comment and a new figure 2 is included in the revised manuscript with the suggested corrections.
Funding Information:	PADI Foundation (47512) — Dr. Valeria C. D'Agostino Fondo para la Investigación Científica y Tecnológica (PICT-2018-02550) — Dr. Valeria C. D'Agostino Northern Arizona University — Dr Loren Buck
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 sixteen SRW fecal samples from live and dead stranded whales in PV from 2013-2018 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 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.

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September 9, 2021

Dr. Joel C. Trexler and Dr. Donovan German
Editor-in-Chief/ Handling Editor
Oecologia

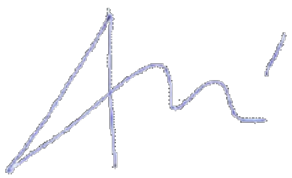
Dear Dr. Trexler and Donovan

Enclosed you will find our revised version of the manuscript "Potential endocrine correlation with exposure to domoic acid in Southern Right Whale (*Eubalaena australis*) at the Península Valdés breeding ground" by Valeria C. D'Agostino, Alejandro Fernández Ajó, Mariana Degrati, Bernd Krock, Kathleen E. Hunt, Marcela Uhart and C. Loren Buck. We thank you very much for the opportunity to resubmit our manuscript to Oecologia and for your kind assistance.

We greatly appreciate the complete comments and suggestions of the Handling Editor and Reviewers that evaluated this manuscript, as they helped to improve its quality and presentation. Our manuscript has thus been modified according to the Reviewers' suggestions.

All changes have been directly inserted in the revised manuscript. Please find below our comments and replies to the points raised by Reviewers.

Yours sincerely,



Dr. Valeria C. D'Agostino

Dr. Alejandro A. Fernández Ajó

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Comments to Authors from Handling Editor

Thank you for submitting to Oecologia. We don't see a ton of whale papers, so this is exciting. As you will see, the reviewers liked the paper overall. They each had suggestions for improving the manuscript. The most common theme was that the authors recognized the small sample size was a problem (but it is a study on Southern Right Whales, not zebrafish!), and they acknowledged it. However, it may need to be acknowledged up front, as soon as the abstract. I don't disagree with this. However, the work is broadly interesting enough to warrant publication after revisions. The other largest request was to perhaps downplay the method validation as a main thrust of the paper. Yes, the validation is important, but is that the focus of the paper, or is it the broader biology and potential problems of DA? Two reviewers thought a little more focus on the biology and a little less on the method validation was warranted. I have to agree since Oecologia has a broad readership. Overall, the suggested changes can be made in a careful revision.

Reply: We greatly appreciate the Handling Editor's comments about our study. Taking these suggestions into account, we have carefully and thoroughly revised our manuscript, including adding a mention of the small sample size to the Abstract, and, in the main text, reducing emphasis on validations in favour of more emphasis on the broader biological questions. We have also either reformulated some sentences or included additional references in order to comply with some specific comments.

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Reply to Reviewer # 1

Reviewer #1: Overall I found very little in this manuscript for which I would suggest edits or revisions, in fact this is one of the best-written manuscripts I have reviewed. I do have a few very minor comments that may or may not need to be addressed with revisions, but that might strengthen the paper somewhat or provide additional clarification. The use of corticosteroids as a HAB-related biomarker is very useful and the description of this method will be of significant importance to researchers doing similar work.

The authors did an excellent job of laying out the background mechanisms of DA production, toxicity and endocrine pathways that can be altered during acute and chronic DA-related stress. The background section was exceptionally well-written and useful to provide all necessary relevant literature and clearly describe the research problem being addressed. The Study Species paragraph in the Methods section seems to be background information, and might be more appropriate to include in the Introduction section instead.

Reply: We greatly appreciate Reviewer # 1's comments. We tested moving the paragraph about SRW natural history to the Introduction as suggested but found that it disrupted the Introduction's focus and flow; we feel that thought the natural history of the study species should be understood by the reader, sometimes a paper flows better if such material is presented in Methods in a paragraph about the study species. Ultimately, we have moved this paragraph back to Methods, but we do appreciate the suggestion.

Reviewer #1: It appears that a Welch's unequal variances t-test was used to compare the concentrations of fGCm between DA-positive and DA-negative groups, but this test assumes normal distributions for both groups. It was unclear if this assumption was true in the case of the DA-positive vs. DA-negative groups, but should be more clearly stated in order to say there was a significant difference between the two, since the DA-positive group only included 3 samples. However, this may be a moot point since the authors suggest but do not conclude that DA is responsible for changes in fGCm, but may also be confounded by other life history factors.

Reply: We agree with Reviewer #1's observation regarding the assumption of normality needed for performing a Welch's t-test, complied with this, in lines 285-288 of the revised manuscript we stated: "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."

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*Reviewer #1: line 447 - "Our results provide the first evidence that HAB-associated neurotoxins such as DA can affect adrenal physiology in whales" - since the authors state that DA *might* be a factor in changes to fGCm levels, I would suggest wording that reflects this, so the reader does not think that the authors are making a conclusion that DA *does* affect fGCm levels. Perhaps changing the sentence to read "...first evidence that HAB-associated neurotoxins such as DA may be a factor affecting adrenal physiology in whales"*

Reply: We agree with this suggestion and have reformulated the sentence as follows: "Our results provide the first evidence that HAB-associated neurotoxins such as DA may affect adrenal physiology in whales." Please see lines 479-480 in our revised manuscript.

Reviewer #1: Table 1 - the authors present DA concentrations ranging between 0.310 and 710 ug/g. It would be useful to provide a bit of reference data for other marine mammal DA values from the literature for comparison. Mainly because the highest reported DA concentration from a marine mammal that I am aware of is from the 2010 Fire et al. paper in Aquatic Mammals, where they reported DA in feces at 258.67 ug/g from a whale stranding during a California mass mortality event co-occurring with a severe Pseudo-nitzschia bloom. The maximum DA value from this manuscript is nearly 3 times greater, but from an individual likely only opportunistically feeding, and in a region not as well-known for severe P-n blooms. I would be interested in hearing the authors thoughts on possible explanations for this, as this is a very exciting finding. Also, this extremely high DA value should be emphasized as a major finding of the paper, and I would suggest including this in the abstract and the discussion.

Well done!

Reply: We greatly appreciate this suggestion and comments about our findings; however, as we explained in our manuscript, the data on positive DA levels in southern right whales were previously published in D'Agostino et al. (2017) (please see lines 139-141. In the present study and in D'Agostino et al. (2017) the DA levels in fecal samples were presented in µg/g of dry weight (by lyophilization), which will result in different levels when compared to DA in non-lyophilized fecal samples. Nearly all published studies for DA in marine mammal fecal samples have used non-lyophilized samples (and thus expressed in wet weight). Therefore, in order to make our data comparable to previous DA-related marine mammal studies, in our previous study, D'Agostino et al. (2017), we have determined the mean water content in fecal whale samples from southern right whales (84%). Therefore, although our DA values were high, considering the water content in fecal samples, our measured values are not the highest reported for a marine mammal species. Nevertheless, our DA values quantified in southern right whales were higher than those reported in feces from North Atlantic right whales on their feeding grounds in the

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Great South Cannel and the Bay of Fundy (Leandro et al. 2010; and for more details see D'Agostino et al. 2017). In addition, the highest levels of DA detected in our study were the highest registered in a living right whale (710 µg DA g^{-1} dry weight or 113.6 µg g^{-1} wet weight assuming a mean water content of 84%); thus, it can be deduced that this whale was exposed to an intense toxic *Pseudo-nitzschia* bloom at the breeding ground of Península Valdés and these data were discussed in D'Agostino et al. (2017). However, following the reviewer's suggestion and for the sake of clarity, we have included information about this as follows: "Three fecal samples from SRWs were positive for DA with levels ranging from 0.30–710 ± 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)." (Please see lines 313-315 and throughout our revised manuscript).

D'Agostino VC, Degrati M, Sastre V, Santinelli N, Krock B, Krohn T, Dans SL, Hoffmeyer MS (2017) Domoic acid in a marine pelagic food web: Exposure of southern right whales *Eubalaena australis* to domoic acid on the Península Valdés calving ground, Argentina. Harmful Algae 68:248-257 https://doi.org/10.1016/j.hal.2017.09.001

Leandro LF, Rolland RM, Roth PB, Lundholm N, Wang Z, Doucette GJ (2010) Exposure of the North Atlantic right whale *Eubalaena glacialis* to the marine algal biotoxin, domoic acid. Marine Ecology Progress Series 398:287–303.

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Reply to Reviewer # 2

Reviewer #2: The authors investigated whether domoic acid (a toxin produced by diatoms) correlated with faecal GC metabolites in southern right whales. In addition, they validated the EIAs for measuring faecal cortisol and corticosterone for this species. Whales with evidence of domoic acid exposure had lower fGCm levels than whales with undetectable domoic acid levels. The authors also report that the highest fGCm level was found in a lactating female without detectable domoic acid levels. The authors acknowledge that their sample sizes were low, but that their study provides preliminary evidence that exposure to domoic acid could alter adrenal function.

The immense effort required to collect this data should be appreciated. I think it can be easy for some readers to be critical of the sample size; however, accessing this type of physiological data from a large, highly mobile, aquatic mammal is phenomenal! The authors have clearly stated the caveats and limitations of the data and have stated that the results are best-suited to serving as a roadmap for future research.

Overall, the manuscript is very well-written. It is clearly and logically set up, and the methodology is detailed. The authors were very careful in using best-practices when performing their hormone metabolite validations and measurements, and this is evident in the methods.

I have a few comments, along with some other minor suggestions, that I hope will be useful.

Reply: We greatly appreciate Reviewer #2's comments and observations about our paper. Taking Reviewer #2's suggestions into account, we have attentively revised our manuscript and have reformulated the text accordingly (please see below).

Reviewer # 2's comment 1: Cohesiveness of objectives and scope: I think there is an opportunity to focus the objectives of the study and increase the fit of the manuscript with a broad ecological journal (as opposed to a journal more focused on physiology, for example). My suggestion would be to set up the objectives to put the focus of the work on (2) and (3) (i.e., state the objectives related to DA as the main goals). This could be followed with a statement that, to accomplish these goals, an enzyme immunoassay was validated to quantify fGCm in faeces of SRW for the first time. Further, in relation to keeping the goals consistent and in-line with title of the paper, quantifying fGCs in lactating females feels a little bit out of place to me as well. However, I agree the data are valuable and should be reported here, I just might consider leaving this out as a core objective of the study unless there is a way to tie it more clearly to the work linking hormones and DA. Finally, I think the paper would benefit from the inclusion of a prediction for how fGCm levels will relate to DA. Although these would be minor changes to the text of the paper, I think it will align the paper more closely with the scope of Oecologia,

while also allowing others who are interested in the finer details of the hormone validations to still access that information easily.

Reply: We agree with the reviewer's comment and have revised the objectives to place the focus of our research on the broader biology and potential problems of DA exposure of relevance for Oecologia readership as follows: "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.", see lines 136-139 of the revised manuscript. We have also excluded the assessment of typical fGCm for lactating females from the main objectives, but we do include it in the results as we consider it valuable data to report here and it aids with data interpretation. Lastly, within lines 76-86 we have included predictions regarding the adrenal response to exposure to DA in the revised manuscript as follows: "As an additional complication, domoic acid is an excitatory amino acid analogue 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 Mahes 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 physiological impacts of exposure to DA."

Reviewer # 2's comment 2: I think it would be useful to provide an estimate of the timeframe faeces will reflect DA levels and fGCs (even if this is an approximation based on other species). This will allow readers to understand what a faecal sample collected from a live or dead animal is reflecting - i.e., does it correspond to a very recent exposure to DA and over what total time period is it likely integrating GC levels.

Reply: Although clearance studies have not been conducted on southern right whales, several studies have documented that DA is cleared rapidly in mammals (Iverson et al. 1989; Truelove and Iverson, 1994; Maucher and Ramsdell 2007). For example, in sea lions it has been estimated that clearance occurs within 48 h of ingestion (Wittmaack et al. 2015). These findings suggest that the southern right whale individuals analysed in our study were exposed to DA recently—likely within a few days. Concerning the fGCm,

clearance studies have not been conducted on southern right whales. 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. Therefore, following the reviewer's suggestion, we have included information about this in our revised manuscript (Lines 173-184 and now reads as follows: "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 SRW's analyzed in our study were exposed to DA recently and the fGCm can be correlated with the DA exposure."

Iverson F, Truelove J, Nera E, Tryphonas L, Campbell J, Lok E (1989). Domoic acid poisoning and mussel-associated intoxication: preliminary investigations into the response of mice and rats to toxic mussel extract. Food and Chemical Toxicology 27(6): 377–384.

Maucher JM, Ramsdell JS (2007) Maternal-fetal transfer of domoic acid in rats at two gestational time points. Environ. Health Perspect. 115:1743-1746.

Millspaugh, JJ, Washburn BE (2004) Use of fecal glucocorticoid metabolite measures in conservation biology research: Considerations for application and interpretation. General and Comparative Endocrinology, 138(3), 189–199. https://doi.org/10.1016/j.ygcen.2004.07.002

Rolland, R. M., Parks, S. E., Hunt, K. E., Castellote, M., Corkeron, P. J., Nowacek, D. P., Wasser, S. K. & Kraus, S. D. (2012). Evidence that ship noise increases stress in right whales. Proceedings of the Royal Society B: Biological Sciences, 279(1737), 2363–2368. https://doi.org/10.1098/rspb.2011.2429

Rolland, R. M., Hunt, K. E., Doucette, G. J., Rickard, L. G. & Wasser, S. K. 2007 The inner whale: hormones, biotoxins and parasites. In The urban whale: North Atlan- tic right whales at the crossroads. (eds S. D. Kraus & R. M. Rolland), pp. 232–272. Cambridge, MA: Harvard University Press.

Truelove J and Iverson F (1994) Serum domoic acid clearance and clinical observations in the cynomolgus monkey and Sprague-Dawley rat following a single IV dose. Bulletin of Environmental Contamination and Toxicology, 52(4):479-486.

Wittmaack C, Lahvis GP, Keith EO, Self- Sullivan C (2015) Diagnosing domoic acid toxicosis in the California sea lion (*Zalophus californianus*) using behavioral criteria: A novel approach. Zoo biology, 34(4):314-320.

Reviewer # 2's comment 3: For the discussion, is there anything else about the three animals exposed to DA that could be leading to the lower GC levels besides lactation? For example, all the individuals were not all sampled at the same time of year and the samples are coming from

a wide year range (2013-2018) - is there any other environmental stressor that could be responsible? It might be worthwhile to state that you do not expect that other environmental pressures differed (or discuss others that could have differed as alternative reasons for the results).

Reply: We greatly appreciate Reviewer #2's suggestion. To address this, we have included a line in the Materials and methods section to clarify that all samples were recovered during the SRW breeding season. It now reads as follows: "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)." (Please see lines 165-167. Additionally, we have included in the discussion more details about possible variables that might influence fGCm levels in different years but, given our inevitably low sample size, we could not address the potential effect any other environmental stressor in the different years of fecal sample collection. The new sentence now reads as follows: "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 nor compare the effects of years considered of high (2003, 2005, 2007–2013), versus low calf mortality (2004, 2006, 2014–2019) (Marón et al. 2021)." Please see lines 426-430

Reviewer # 2's comment 4: The authors suggest a number of important future research goals. Given the sample size, I think this is a really important goal of the overall paper, as stated in the introduction (i.e., that the data is a preliminary assessment to identify avenues for further research). As a result, I think the discussion should have a strong section on future work. The authors mention in the conclusion that other physiological traits could be measured. Perhaps this could be moved up to the main part of the discussion and there could be a bit more detail as to why those traits in particular could be useful (i.e., what extra information will be garnered from aldosterone, thyroid hormones, etc.) and whether any other traits related to other aspects of physiological function could be useful (e.g., metrics related to immune function or reproduction given the sublethal impacts outlined in the introduction). In addition, what is needed in terms of further research to allow these types of analyses to benefit the conservation of SRW (i.e., how can the preliminary results presented in the manuscript best be scaled up to give more conclusive evidence that DA is a threat to the species)?

Reply: We appreciated reviewer #2's suggestion and have thus included in our revised version of the manuscript a new section "Future Directions" in which we aim to address all the suggestions made by Reviewer #2's comment 4. The new section now reads as follows: "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." Please see the new section in lines 493-510.

Minor comments:

Line 84-85 - I am wondering if you could add an additional sentence about the mechanism behind lowered GCs in response to DA to give an indication of why the HPA axis might be suppressed instead of activated? This would also lead to greater understanding by readers before providing a prediction for SRW.

Reply: **We appreciate the reviewer's suggestion. In lines 76-86 we have stated that we could predict that fGCm would rise during DA exposure as a generalized response to DA-related illness. However, we also referenced a previous study (Gulland et al. 2012) which has reported the opposite correlation in sea lions exposed to DA, which could be explained if DA exposure lead to adrenal function insufficiency. We opened this paragraph also mentioning that DA is an excitatory amino acid analogue of glutamate, which in turn is an excitatory amino acid that plays an important role in the activation of the HPA axis, regulating many pituitary hormones involved in the stress response. We think that the information provided in these lines is based on the best current knowledge. When exploring this correlation we could not predict a directionality in the regulation of the HPA axis, but we considered the two alternatives as possible outcomes.**

Line 162 - Can you please add the range of months in which the samples were collected.

Reply: Taking reviewer #2's suggestion into account, we modified the sentence as follows: "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)." Please see lines 165-167 of the revised manuscript.

Line 270 - Whales were divided into categories of either a detectable or undetectable level of DA. Perhaps this could be stated above in the domoic acid methods section as I had to scroll back up to make sure I understood how this was quantified. For example, at Line 192 you could indicate that DA was measured as ug/g, but whales were classified as having either detectable or non-detectable levels.

Reply: Addressing reviewer #2's suggestion, we have made the following clarification in the Materials and methods section lines 281-283 "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."

Line 417 - "GCs" should be "GC"

Reply: Complied with. We greatly appreciate Reviewer #2's observation. Please see line 449 of the revised manuscript.

Line 428 - add the word "or"

Reply: Complied with. The sentence has been corrected as indicated by Reviewer # 2. Please see line 460 of the revised manuscript.

Line 436 - "individuals" should be "individual"

Reply: Complied with. "individuals" has been replaced by "individual". Please see line 468 of the revised manuscript.

Table 1 - This table may be easier to read if there is space to use full label headings of corticosterone and cortisol metabolites instead of B and F.

Reply: Taking reviewer # 2's suggestion into account, we have reformulated table 1 accordingly. Please see Table 1 in our revised manuscript.

Figure 2 - I suggest larger axis labels and legends to make the graphs easier to read.

Figure 3 - Open circles (rather than solid black dots) might display the data more clearly here.

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Reply: Taking reviewer # 2's suggestions into account, we have reformulated figures 2 and 3, and the supplementary material accordingly. Please see Figures 1 and 3, and the supplementary figures Fig.S1 and Fig.S2 in our revised manuscript.

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Reply to Reviewer # 3

Reviewer #3: General Comments

The authors present a well-written and methodologically thorough study examining the levels of fecal glucocorticoid metabolites (fGCm) in the Southern Right Whale (SRW) and the potential relationship of fGCm levels with exposure to the HAB-produced neurotoxin, domoic acid (DA).

Reply: We greatly appreciate Reviewer #3's comments and observations about our paper. We have attentively revised our manuscript and have reformulated the text taking Reviewer #3's suggestions into account and have replied to the specific comments and questions below.

The key statement in the Discussion section of the manuscript is found on lines 388-398: "Due to low sample size, we cannot fully separate the effect of DA from the possible effect of lactation." Hence, the authors have correctly indicated in their title the "potential" endocrine correlation with DA exposure. I understand and appreciate the difficulty associated with obtaining these types of samples and thus the very small sample size. Accordingly, the authors have not overstated their conclusions, but have provided some very interesting and valuable data that can be built on by similar continuing efforts in the future. I might suggest that the authors refer briefly, but more specifically, to this potential caveat in the abstract.

Reply: We greatly appreciate that Reviewer #3 recognizes the difficulties associated with obtaining these types of samples. In lines 50-52 of the abstract, we now acknowledge this limitation as follows: "Though 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."

In terms of methods, it would have been highly beneficial if the authors had conducted spike-recovery experiments in order to assess the efficiency of the extraction protocol employed here. As noted below in the Specific Comments, heat produced by the 15 min sonication step may have caused some degradation of the target hormones (I am unsure as to the temperature stability of fGCm); however, spike-recovery experiments would aid in determining if this is a potential problem.

Reply: We greatly appreciate Reviewer #3's comments and suggestions. However, percentage-recovery of added radiolabeled hormone was not tested in this study, since this method of testing extraction efficiency is not applicable to solid-tissue sample types such as feces, shed skin, hair, baleen, etc. This is because the added radiolabelled hormone does not behave like native hormone, as Palme et al. state in their 2013 review. It is not

possible to mix liquid parent hormone into a solid sample type in a way that adequately mimics the nature of deposition of native hormone into the gut and the fecal matrix. Native hormone and hormone metabolites are presumably bound into the solid particles of dried fecal sample; added radiolabelled hormone could only loosely adhere to the surface of the dried particles and thus would be more easily extracted. Further, for feces in particular, an additional, and major, issue is that fecal steroid hormone metabolites are not chemically identical to the parent hormone that circulates in plasma. For example, in the closely related North Atlantic right whale, HPLC analyses indicate that circulating cortisol is metabolized to a complex of at least nine different immunoreactive fGCms (Hunt et al. 2006) — none of which are commercially available in the radiolabelled form that would be necessary to do spike experiments. For these reasons, spike experiments to assess percentage recovery are generally not advised for mammalian fecal hormone studies (Palme 2019). It is for this reason that fecal endocrinology, as a field, tends to focus on relative patterns rather than absolute concentrations. The most relevant question, in our view, is: do the relative patterns of hormones (i.e., regardless of the % recovered) reflect the physiological state of the animal? If so, the assay has predictive and explanatory utility, regardless of % recovery. If not, it is not a useful method, regardless of % recovery. For this reason, our analysis focuses on relative patterns and not on absolute concentrations. We have included a sentence in the Materials and methods section clarifying that % recovery was not tested and adding a citation to reviews that justify this course of action. Please see lines 232-236 "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."

Hunt KE, Rolland RM, Kraus SD, Wasser SK (2006) Analysis of fecal glucocorticoids in the North Atlantic Right Whale (Eubalaena glacialis). General and Comparative Endocrinology 148(2):260-272.

Palme R, Touma C, Arias N, Dominchin MF, Lepschy M (2013) Steroid extraction: get the best out of faecal samples. Wien Tierarztl Monatsschr 100(9-10):238-46

Palme R (2019) Non-invasive measurement of glucocorticoids: advances and problems. Physiology & behavior 1;199:229-43.

Another, potentially more important methods issue is that the authors' 'matrix effect test' did initially show a matrix effect, which was then corrected by employing SPE; however, it was not stated whether the pooled, serially diluted SRW fecal extract used in the 'parallelism' test (line 228) was also subjected to SPE clean-up. If so, there is not an issue, but if not, then the dilution

is serving to dilute both the target analyte and the matrix, thereby complicating interpretation of the results. From their matrix test data, it seems that an extract dilution of 1:5 (or less) and perhaps more, does cause a matrix effect in the EIAs, thereby requiring SPE to eliminate this effect. Use of SPE in the parallelism test should be clarified in the text. Overall, the authors have presented a solid and valuable piece of work, even given the fact that they had few samples to work with as a result of the immense challenges associated with obtaining fecal samples on the SRW calving grounds, where defecation is very limited. The data analysis is thorough and the discussion of the results is tempered appropriately, given the very small sample size. As noted above, the findings presented here should be valuable in informing future efforts to better understand the potential effects of domoic acid on the SRW population, which is extremely challenging to study in a non-invasive manner.

Reply: We greatly appreciate Reviewer #3's comments and observations about our paper. We have carefully revised our manuscript and have reformulated the text taking Reviewer #3's suggestions into account. Please see our responses to the specific comments and questions below for more details.

Specific Comments

Line 60: replace 'Broadwate' with 'Broadwater'; and correct reference to read: Broadwater MH, Van Dolah FM, Fire SE (2018) Vulnerabilities 542 of marine mammals to harmful algal blooms. Harmful Algal Blooms: A Compendium Desk Reference. John Wiley & Sons, Ltd. Pp. 191-222

Reply: Complied with. Following the reviewer's observation, we have amended this mistake in the revised version of our manuscript. Please see line 61 and references in our revised manuscript.

Line 175: it would be good to understand what comprises 'lysing matrix D', if not proprietary; at least some sense of what this matrix contains, if the exact formulation is not available

Reply: Taking Reviewer # 3's comment into account, we added a description of lysing matrix D and the sentence now reads: "…FastPrep tubes containing 0.9 g ceramic beads (lysing matrix D, Thermo Savant, Illkirch, France),…" Please see lines 189-190 in our revised manuscript.

Line 186: it is not apparent from the D'Agostino reference whether effects of the fecal matrix were taken into account for the calibration of the LC-MS/MS analysis; accuracy of DA quantification could definitely be affected by the fecal matrix - how did the authors address this uncertainty if potential (likely) matrix effects were not accounted for?

Reply: Matrix effects that possibly could influence DA values were not considered in this study. This decision was taken due to the low availability and high price of DA standards, but more importantly accurate DA determinations were outside the scope of this work as to date nothing is known about the relationship of DA levels in fecal pellets and the health status of marine mammals. In this study, the aim was to investigate a possible correlation between DA and hormone levels and a potential systematic error in DA values would not affect the results of the study.

Line 187: it is not clear why the authors chose to exhaustively extract the material 20 times - this seems quite excessive! Was there a rationale or precedent for this?

Reply: We do not have an explanation for the phenomenon of the retention of DA in lyophilised fecal samples. DA concentrations after repeated extraction steps were low, but still clearly detectable. Surprisingly after 11-20 extraction steps DA levels did not show a gradual decrease from one extraction step to the next. We also cannot say anything about recovery, because adding DA to fecal pellets under these circumstances would never result in representative recoveries, as it is well known that added analytes show far better extractabilities than analytes that have to be released from the sample matrix.

Line 191: the authors should provide some indication of what this 'correction factor' was, which appears to have been based on the results from extractions 3-20; the statement that these extractions contained only 'minor additional DA' (line 188) is not sufficiently clear to justify a 'correction factor' based on such results being applied to their analytical data.

Reply: The correction factor was calculated on based that of the total DA of the 20 extractions in the sample BFA9 (which presented high values of DA that fell outside the calibration range), the 38% were found in from 3rd to 20th extractions. Therefore, based on this finding, the two other samples (BFA11, BFA13) positives to DA were only extracted twice, with final DA content estimated as the sum of DA in the first extract and second extract, plus the correction factor of 0.38 based on data from the 3rd-20th extractions of sample BFA9. Considering the observations made by Reviewer # 3, we have now included information about the correction factor in our revised manuscript as follows: "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). In order 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.

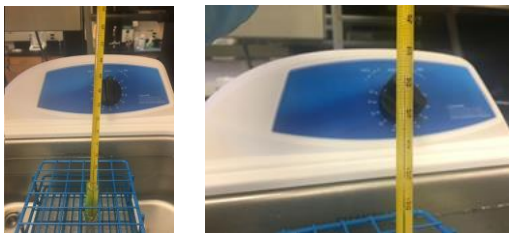
Domoic acid levels were expressed as µg g^{-1} dry fecal sample." Please see lines 202-208 in the revised manuscript

Lines 191-192: the authors need to provide the limit of detection (LOD) and limit of quantification (LOQ) for their LC-MS/MS method

Reply: Complied with. We have specified the limit of detection (LOD) and limit of quantification (LOQ) for our LC-MS/MS method. Please see the caption of Table 1 in the revised version of our manuscript.

Line 214: sonication, especially probe sonication (although it is not clear whether probe or bath sonication was used here), can generate considerable heat; did the authors monitor temperature in this extract and/or attempt to control the temperature in order to avoid potential degradation of the target hormones?

Reply: Thanks for this observation. In this study we utilized a bath sonication. We have included details in the revised manuscript clarifying the type of sonication and equipment used as follow: "…bath sonicated for 15 min (Branson 3800 ultrasonic cleaner)…" Please see lines 230-231 Bath sonication for 15 min does not produce significant heat as tubes are submerged through the process in room temperature water (approximately 20°C). We have monitored the temperature inside a glass tube before and after 15 min bath sonication and have not perceived a significant change in the temperature (Please see photographs below).



Temp before 21°C (left) and after 23°C (right) sonication.

Line 223: is it known whether other fecal metabolites unrelated to GCs may have epitopes recognized by antibodies employed in the two kits tested?

Reply: Thank you for your comment. We refer Reviewer 3 to the following tables for #K014 corticosterone (top) and #K003 cortisol (bottom) kits cross reactivity reported by the manufacturer Ann Arbor, MI, USA. We believe that mentioning the specific kits used and manufacturer should provide enough information for the readers regarding the specificity and cross reactivity of the assays used in this study.

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CROSS REACTIVITY

The following cross reactants were tested in the assay and calculated at the 50% binding point.

Steroid	Cross Reactivity (%)	Steroid	Cross Reactivity (%)
Corticosterone	100%	Testostrone	0.03%
1-dehydrocorticosterone	18.90%	Corticosterone-21-hemisuccinate	< 0.1%
Desoxycorticosterone	12.30%	Cortisone	< 0.08%
1α-hydroxycorticosterone	3.3%	Estradiol	< 0.08%
11-dehydrocorticosterone	2.44%	17-hydroxyprogesterone	< 0.01%
Tetrahydrocorticosterone	0.76%	Allopregnanolone	< 0.01%
Aldosterone	0.62%	Dehydroepiandrosterone sulfate	< 0.01%
Cortisol	0.38%	Estrone-3-glucuronide	< 0.01%
Progesterone	0.24%	Estrone-3-sulfate	< 0.01%
Dexamethasone	0.12%		

CROSS REACTIVITY

The following cross reactants were tested in the assay and calculated at the 50% binding point.

Steroid	Cross Reactivity (%)
Cortisol	100%
Dexamethasone	18.8%
Prednisolone (1-Dehydrocortisol)	7.8%
Corticosterone	1.2%
Cortisone	1.2%
Progesterone	< 0.1%
Estradiol	< 0.1%
Cortisol 21-Glucuronide	< 0.1%
1α-hydroxycorticosterone	< 0.1%
Testosterone	< 0.1%

Lines 278-280: it does appear that the diluted pool extract shows two distinct segments to the 'curve', with dilutions 1:1 through 1:4 being of higher slope than for dilutions 1:4 through 1:16. The latter looks (by eye) to be more in line with the standards curve - again, relating to my question above, were these dilutions of the pool extract subjected to SPE clean-up? This needs to be clarified. If SPE was not done, this could explain the better agreement in slope with dilutions greater than 1:5.

Reply: We much appreciate reviewer #3's comment. In compliance with the reviewer's comments and suggestions we have make amendments in the revised manuscript as described below.

In the parallelism test, an F statistic test is used 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. It now reads in the revised manuscript as follows: "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." (Please see lines 275-277 in the Materials and methods section).

With regards to the parallelism test performed with SPE, we appreciate the reviewer's observation and have addressed this issue. In the revised version, we reported the parallelism test performed after SPE, and we have made the necessary clarifications throughout the text specifying that SPE extractions where used to perform the validations. In the revised manuscript Materials and methods section's lines 239-240 we now state: "…corticosterone and cortisol metabolites in SRW fecal solid phase extracts (SPE)." whereas in the Results' section it now reads: "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)." please see lines 308-311 and also this was clarified in figure's #2 legend.

- *Line 288: it is not clear whether the error term applies only to the 710 value (see line 291, where the same value is given for the error term with the 710 value; also, the same is true in Table 1)*

Reply: Taking Reviewer # 3's comment into account, we have clarified this as follow: "Three fecal samples from SRWs were positive for DA with levels ranging from 0.30–710 ± 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)." Please see lines 313-315 in the revised version of our manuscript.

Our reply to Reviewer # 3's comment: *this the only DA value of the three positive samples with an associated error term, presumably based on the three 'technical replicates' referred to on line 186? Why were two additional aliquots of the other positive samples not also extracted in order to provide comparable replication and error terms?* **Yes, Reviewer #3 is right, the DA level from BFA9 is the only DA value with an associated error term due the methodology applied for DA extraction from the fecal sample. In this study we did not aim to determine the absolute DA values in SRW fecal samples, but instead we aimed to**

determine if DA was detectable or not detectable and assess the correlation with fGCm. The absolute values in this context do not yield relevant information for the work; therefore, the effort and costs associated to generating them was deemed not justifiable.

Figures

Lines 817-821 (Fig. 2 legend): the individual graphs need to be labelled with A, B, C, or D. Both x- and y-axes on all graphs require the addition of titles and units.

Reply: Thanks for this observation, we have addressed this comment and a new figure 2 is included in the revised manuscript with the suggested corrections.

For publication in *Oecologia*

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

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Valeria C. D'Agostino and Alejandro Fernández Ajó contributed equally to this work.

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.

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 sixteen SRW fecal samples from live and dead stranded whales in PV from 2013-2018 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 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

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 analogue 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 Mahes 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

85 exposure to DA could lead to an adrenal function insufficiency (Gulland et al. 2012). Overall,
86 GC levels are a potential useful metric for assessing physiological impacts of exposure to DA.

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

106 The southwestern Atlantic southern right whale (SRW, *E. australis*) population that breeds
107 off Península Valdés, Argentina, migrates annually from their feeding ground and remains in
108 the region of Península Valdés during the austral winter and spring months. In Península
109 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 subantarctic 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 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 Subantarctic (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

158 are the norm for this species, and twinning has never been observed. Lactation duration is
159 poorly known for this species (Best et al. 2015). Some calves have been seen feeding on
160 zooplankton patches next to their mothers at an estimated age of six months (Best 2007). In
161 other cases, females return to coastal breeding areas in the year following birth, still
162 accompanied by their yearling calf; therefore, if these calves are still nursing, lactation would
163 have lasted about 12 months (Thomas and Taber 1984; Best et al. 2003, 2015).

164 **Fecal sample collection**

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

183 that the SRW's analyzed in our study were exposed to DA recently and the fGCm can be
184 correlated with the DA exposure.

185 **Domoic acid determination**

186 Southern right whale feces were lyophilized to remove water (OPERON FDU-8606, Korea)
187 and stored at -20 °C until DA was extracted following D'Agostino et al. (2017). Briefly, fecal
188 samples were thawed at room temperature and ~10 mg aliquots (± 0.001%; BA110S, Sartorius,
189 Göttingen, Germany) transferred to FastPrep tubes containing 0.9 g ceramic beads (lysing
190 matrix D, Thermo Savant, Illkirch, France), and 1 ml methanol was added, homogenized by
191 reciprocal shaking at maximum speed (6.5 m s^{-1}) for 45 s in a Bio101 FastPrep homogenizer
192 (Thermo Savant, Illkirch, France), and centrifuged at 16,100 × *g* at 4 °C for 10 min.
193 Supernatants were transferred to spin-filters (0.45 µm pore-size; Millipore Ultrafree, Eschborn,
194 Germany) and centrifuged at 16,100 × *g* at 4 °C for 5 min. Resulting filtrates were transferred
195 to autosampler vials; the final volumes were adjusted to 1 ml with methanol and stored at 4 °C
196 until use. The residues of each fecal sample in the FastPrep tubes were re-extracted once as
197 described above. Accordingly, two extracts were obtained for each fecal sample, and both were
198 analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)
199 separately (for protocol details of the LC–MS/MS analyses, see D'Agostino et al. 2017). One
200 sample (BFA9, see Table 1) presented high values of DA that fell outside the calibration range.
201 This sample was divided into three aliquots, with each consecutively extracted 20 times (n =
202 60). Most DA in this sample was determined in the first two extracts, with the 3^{rd}-20^{th}
203 extractions yielding only minor additional DA (38% of the sum of the first two extracts). In
204 order to avoid the very time-intensive multiple extractions, the two other samples (BFA11,
205 BFA13) with high DA levels were only extracted twice, with final DA content estimated as the
206 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 was comprehensively cleaned with 70% ethanol. Weighed fecal samples were placed in 16x100 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 13x100 mm borosilicate glass tube. 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

231 ultrasonic cleaner), vortexed for 15 min, transferred to 1.5 ml vapor proof o-ring-capped
232 cryovials, and stored at -80 °C until assay within one week. Percentage recovery was not
233 evaluated in this study, as it is not possible to mimic behavior of native hormone in non-plasma
234 sample types via addition of liquid radiolabelled parent hormone, particularly given that fecal
235 hormone metabolites are not chemically identical to the parent hormone (Palme et al. 2013);
236 rather, data analysis focuses on relative patterns and not on absolute concentrations.

237 **Hormone assays and validation**

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

247 We conducted tests of parallelism and accuracy using standard methods described in
248 Grotjan and Keel (1996). To test for parallelism, a pooled SRW fecal SPE extract was serially
249 diluted in assay buffer to produce eight dilutions (range 1:1 – 1:128) which were then assayed
250 as unknowns in both the cortisol and corticosterone EIAs, following which the slope of
251 percentage of bound antibody vs. relative dose was compared to the slope of the known-
252 concentration standards. Parallelism of the two binding curves (serially diluted pool and
253 hormone standards) indicates that the antibody binds well to an immunoreactive component in
254 the sample of interest, with very similar affinity as to pure parent hormone; this is considered
255 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 re-analyzed. 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 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 0.30–710 ± 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 (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 ($p<0.05$; Fig. 3). Exclusion of samples from dead whales did not change direction or significance of these results ($p<0.05$; 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 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 fifteen of sixteen 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 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;

401 Corkeron et al. 2017; Valenzuela-Molina et al. 2018). In fact, in the present study, the highest
402 fGCm levels were registered in a lactating female that was sighted with two calves (BFA20;
403 Online Resource Fig. S3). Right whales typically give birth to a single calf every 3-5 years after
404 a 12–13-month gestation period (Best 1994; Kraus and Hatch 2001), and observations of twin
405 births in right whales (i.e., associated with genetic evidence) are unknown (Best et al. 2015). In
406 the present study, both calves with BFA20 showed normal behaviors and appeared to be in
407 good condition (Best et al. 2015). However, we perceived that the female tried to avoid the
408 larger calf (Calf 2 in Online Resource Fig. S3). An instance of genetically confirmed calf-
409 swapping has been documented in NARW, in which two mothers exchanged calves shortly
410 after birth and then each nursed the "wrong" calf for months afterwards (Frasier et al. 2010).
411 Thus, it is possible for a right whale calf to follow the wrong mother, and for a right whale
412 mother to nurse a calf that is not her own (Frasier et al. 2010). Irrespective of whether this
413 female was the mother of both calves, or if both calves were nursing, it is likely that the resulting
414 energetic burden and physiological stress could have resulted in elevated fGCm levels in this
415 female.

416 Due to low sample size, we cannot fully separate the effect of DA from the possible effect
417 of lactation. Most of our non-DA-detected samples were from lactating females, and since
418 lactating females tend to have higher fGCm, the higher fGCm noted in the non-DA-detected
419 group may be partially due to the confounding effect of lactation. In this study, low *n* precluded
420 use of statistical models that could separate the effects of DA exposure and lactation. However,
421 the scale of the difference in fGCm noted here exceeds the typical effect of lactation on fGCm
422 in large whales. For example, in NARWs, lactating females have fGCm levels that are only
423 mildly elevated compared to non-lactating females, similar to fGCm of immature males, and
424 lower than fGCm of pregnant females and mature males. Thus, we suggest that the large
425 difference in fGCm noted here between the DA and non-DA-detected groups is most likely

426 attributable to the effect of DA exposure rather than lactation. Similarly, due to our inevitable
427 low sample size, we could not separate the effect of potential environmental or oceanographic
428 conditions prevailing in the different years nor compare the effects of years considered of high
429 (2003, 2005, 2007–2013), versus low calf mortality (2004, 2006, 2014–2019) (Marón et al.
430 2021). Greater sample sizes will be necessary to conclusively answer these questions, and we
431 encourage cetacean fecal hormone researchers to include toxicological analyses where possible,
432 and to archive samples of feces for future analyses.

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

447 The fecal samples collected from the two live calves had similar fecal corticosterone
448 metabolite levels and calf BFA19 was the only whale whose fecal cortisol metabolite levels
449 were higher than the corticosterone metabolites. Several studies have suggested that the GC
450 levels in baleen whale calves are highly variable because they may ingest maternal hormones

451 through milk and then concentrate them in their feces (Hunt et al. 2006, 2019; Fernández Ajó
452 et al. 2018); in this study, both calf samples were roughly in the middle of the range seen in
453 other individuals, but more calf samples will be necessary to determine how fGCm levels of
454 nursing calves compare to those of non-nursing animals.

455 In the present study, three fecal samples of deceased individuals were recovered at necropsy
456 from stranded SRW, one from a juvenile male and the other two from unknown sex individuals.
457 Whale BFA6 was a juvenile male necropsied by the Southern Right Whale Health Monitoring
458 Program on October 5th, 2014 (100514PV-Ea18; for details see Alzugaray et al. 2020).
459 Unfortunately, no data associated with the dead whales in 2013 (BFA1 and BFA2) such as sex,
460 age-class, photographs or health condition were registered. Therefore, we cannot know if the
461 fGCm values recorded in these whales might be attributable to exposure to stressors
462 experienced before death. In NARW, fGCm levels from samples collected at necropsy correlate
463 with cause of death, with entanglement in fishing gear associated with higher fGCm than
464 sudden death due to vessel strike. In extreme chronic stress (e.g., illness, anthropogenic
465 disturbances, exposure to phycotoxins during HABs), the HPA axis can begin to fail, with
466 circulating GC levels eventually declining below normal. Consequently, physiological state
467 may be altered, immune system function depressed, normal activities such as feeding,
468 reproduction, lactation disrupted and, thus, the survival of the individual is reduced (Dickens
469 and Romero 2013; Rolland et al. 2017). Several experiments in stress physiology, both in the
470 laboratory and the field, have reported that declines in GCs often occur under prolonged or
471 repeated exposure to chronic stressors (Rich and Romero 2005; Dickens and Romero 2013). In
472 SRW calves and in humpback whales (*Megaptera novaeangliae*) exposed to chronic stress,
473 baleen GC content rises gradually for weeks as health declines, but then falls sharply just prior
474 to death as individuals become moribund (Fernández Ajó et al. 2018; Gabriele et al. 2020). The
475 three cases presented here will hopefully be the start of a growing dataset to which other

476 researchers can add, ultimately enabling future analyses that can further investigate whether
477 fGCm in samples collected from necropsies of SRWs can illuminate cause of death.

478 ## Conclusions

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

492 ## Future directions

493 Traditional endocrinological methods of analysis include blood sampling from individuals,
494 but this is not possible for large whales (Hunt et al. 2013). Therefore, sampling and analysis of
495 non-traditional matrices such as feces, respiratory vapor, and blubber in combination with
496 collection of samples of baleen, earplugs, and feces from dead individuals would likely increase
497 sample sizes and thus our understanding of the interrelationships among DA exposure and age,
498 sex, and reproductive status of cetaceans. Given that chronic exposure to DA could alter the
499 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.

References

Alfonso M, Duran R, Arufe MC (2000) Effect of excitatory amino acids on serum TSH and thyroid hormone levels in freely moving rats. Horm Res 54(2):78-83 https://doi.org/10.1159/000053236

Alzugaray L, Di Martino M, Beltramino L, Rowntree VJ, Sironi M, Uhart MM (2020) Anthropogenic debris in the digestive tract of a southern right whale (*Eubalaena australis*) stranded in Golfo Nuevo, Argentina. Mar Pollut Bull 161:111738 https://doi.org/10.1016/j.marpolbul.2020.111738

Arufe MC, Arias B, Duran R, Alfonso M (1995) Effects of domoic acid on serum levels of TSH and thyroid hormones. Endocr Res 21(3):671-680 https://doi.org/10.1080/07435809509030482

Ayres KL, Booth RK, Hempelmann JA, Koski KL, Emmons CK, Baird RW, Balcomb-Bartok K, Hanson MB, Ford MJ, Wasser SK (2012) Distinguishing the impacts of inadequate prey and vessel traffic on an endangered killer whale (*Orcinus orca*) population. PLoS One 7. doi:10.1371/journal.pone.0036842

Bastida R, Rodríguez D (2009) Ballena franca austral [Southern right whale]. In: Vázquez Mazzini (ed) Mamíferos marinos de Patagonia y Antártida [Marine mammals of Patagonia and Antarctica]. Buenos Aires, Argentina: Zagier & Urruty Publications pp. 72-84

Best PB (1994) Seasonality of reproduction and the length of gestation in southern right whales *Eubalaena australis*. Proc Zool Soc Lond 232:175-189 https://doi.org/10.1111/j.1469-7998.1994.tb01567.x

Best PB, Schaeff CM, Reeb D, Palsbøll P (2003) Composition and possible function of social groupings of southern right whales in South African waters. Behaviour 140:1469-1494 https://doi.org/10.1163/156853903771980675

569 Best PB (2007) Whales and dolphins of the southern African subregion. Cambridge University
570 Press, Cape Town, South Africa

571 Best PB, Elwen SH, Palsbøll PJ, Thornton M, Austin E, Vinding K (2015) Possible non-
572 offspring nursing in the southern right whale, *Eubalaena australis*. J Mammal 96(2):405-
573 416 https://doi.org/10.1093/jmammal/gyv042

574 Bonier F, Moore IT, Martin PR, Robertson RJ (2009) The relationship between fitness and
575 baseline glucocorticoids in a passerine bird. Gen Comp Endocrinol 163:208-213
576 https://doi.org/10.1016/j.ygcen.2008.12.013

577 Brann DW, Mahesh VB (1994) Excitatory amino acids: function and significance in
578 reproduction and neuroendocrine regulation. Front Neuroendocrinol 15:3-49
579 https://doi.org/10.1006/frne.1994.1002

580 Broadwater MH, Van Dolah FM, Fire SE (2018) Vulnerabilities of marine mammals to harmful
581 algal blooms. Harmful Algal Blooms. John Wiley & Sons, Ltd. 2:191-222

582 Brodie EC, Gulland FMD, Greig DJ, Hunter M, Jaakola J, Leger J.St. Leighfield TA, Van
583 Dolah FM (2006) Domoic acid causes reproductive failure in California sea lions (*Zalophus*
584 *californianus*). Mar Mamm Sci 22(3):700-707 doi: 10.1111/j.1748-7692.2006.00045.x

585 Core Team R (2017) R: A language and environment for statistical computing. R Foundation
586 for Statistical Computing, Vienna, Austria

587 Corkeron P, Rolland RM, Hunt, KE, Kraus SD (2017) A right whale pootree: classification
588 trees of faecal hormones identify reproductive states in North Atlantic right whales
589 (*Eubalaena glacialis*). Conserv Physiol 5(1):cox006
590 https://doi.org/10.1093/conphys/cox006

591 Crespo EA, Pedraza SN, Dans SL, Svendsen GM, Degrati M, Coscarella MA (2019) The
592 southwestern Atlantic southern right whale, *Eubalaena australis*, population is growing but
593 at a decelerated rate. Mar Mam Sci 35(1):93-107 doi: 10.1111/mms.12526

594 D'Agostino VC, Hoffmeyer MS, Almandoz GO, Sastre V, Degrati M. 2015. Potentially toxic
595 *Pseudo- nitzschia* species in plankton and fecal samples of *Eubalaena australis* from
596 Península Valdés calving ground, Argentina. J Sea Res 106:39-43
597 https://doi.org/10.1016/j.seares.2015.09.004

598 D'Agostino VC, Hoffmeyer MS, Degrati M (2016) Faecal analysis of southern right whales
599 (*Eubalaena australis*) in Península Valdés calving ground, Argentina: *Calanus australis*, a
600 key prey species. J Mar Biol Assoc UK 96(4):859-868 doi:10.1017/S0025315415001897

601 D'Agostino VC, Degrati M, Sastre V, Santinelli N, Krock B, Krohn T, Dans SL, Hoffmeyer
602 MS (2017) Domoic acid in a marine pelagic food web: Exposure of southern right whales
603 *Eubalaena australis* to domoic acid on the Península Valdés calving ground, Argentina.
604 Harmful Algae 68:248-257 https://doi.org/10.1016/j.hal.2017.09.001

605 D'Agostino VC, Degrati M, Santinelli N, Sastre V, Dans SL, Hoffmeyer MS (2018) The
606 seasonal dynamics of plankton communities relative to the foraging of the southern right
607 whale (*Eubalaena australis*) in northern Patagonian gulfs, Península Valdés, Argentina.
608 Cont Shelf Res 164:45-57 https://doi.org/10.1016/j.csr.2018.06.003

609 Dickens MJ, Romero LM (2013) A consensus endocrine profile for chronically stressed wild
610 animals does not exist. Gen Comp Endocrinol 191:177-189
611 https://doi.org/10.1016/j.ygcen.2013.06.014

612 Erdner DL, Dyble J, Parsons ML, Stevens RC, Hubbard KA, Wrabel ML, Moore SK, Lefebvre
613 KA, Anderson DM, Bienfang P, Bidigare RR, Parker MS, Moeller P, Brand LE, Trainer

VL (2008) Centers for Oceans and Human Health: a unified approach to the challenge of harmful algal blooms. Environ Health 7,S2 https://doi.org/10.1186/1476-069X-7-S2-S2

Fernández Ajó AA, Hunt KE, Uhart M, Rowntree V, Sironi M, Marón CF, Di Martino M, Buck CL (2018) Lifetime glucocorticoid profiles in baleen of right whale calves: potential relationships to chronic stress of repeated wounding by Kelp Gull. Conserv Physiol 6: coy045 https://doi.org/10.1093/conphys/coy045

Fernández Ajó AA, Hunt KE, Giese AC, Sironi M, Uhart M, Rowntree VJ, Marón C, Dillon D, DiMartino M, Buck CL (2020) Retrospective analysis of the lifetime endocrine response of southern right whale calves to gull wounding and harassment: a baleen hormone approach. Gen Comp Endocrinol 113536 https://doi.org/10.1016/j.ygcen.2020.113536

Fire SE, Wang Z, Berman M, Langlois GW, Morton SL, Sekula-Wood E, Benitez-Nelson CR (2010) Trophic transfer of the harmful algal toxin domoic acid as a cause of death in a minke whale (*Balaenoptera acutorostrata*) stranding in southern California. Aquat Mamm 36(4):342-350 doi: 10.1578/AM.36.4.2010.342

Fire SE, Bogomolni A, DiGiovanni Jr RA, Early G, Leighfield TA, Matassa K, Miller GA, Moore KM, Moore M, Niemeyer M, Pugliares K (2021) An assessment of temporal, spatial and taxonomic trends in harmful algal toxin exposure in stranded marine mammals from the US New England coast. PLOS ONE 16(1): e0243570 https://doi.org/ 10.1371/journal.pone.0243570

Frasier TR, Hamilton PK, Brown MW, Kraus SD, White BN (2010) Reciprocal exchange and subsequent adoption of calves by two North Atlantic right whales (*Eubalaena glacialis*). Aquat Mamm 36(2):115-120 doi: 10.1578/AM.36.2.2010.115

French SS, McLemore R, Vernon B, Johnston GIH, Moore MC (2007) Corticosterone modulation of reproductive and immune systems trade-offs in female tree lizards: long-

638 term corticosterone manipulations via injectable gelling material. J Exp Biol 210:2859-
639 2865 https://doi.org/10.1242/jeb.005348

640 Gabriele CM, Taylor LF, Huntington KB, Buck CL, Hunt KE, Lefebvre KA, Lockyer C, Lowe
641 C, Moran JR, Murphy A Rogers MC, Trumble SJ, Raverty S (2020). Humpback whale
642 #441 (Festus): Life, death, necropsy, and pathology, Natural Resource Report
643 NPS/GLBA/NRR.

644 Goldstein T, Zabka TS, DeLong RL, Wheeler EA, Ylitalo G, Bargu S, Silver M, Leighfield T,
645 Van Dolah F, Langlois G, Sidor I, Dunn JL, Gulland FMD (2009) The role of domoic acid
646 in abortion and premature parturition of California sea lions (*Zalophus californianus*) on
647 San Miguel Island, California. J Wildl Dis 45(1):91-108 https://doi.org/10.7589/0090-
648 3558-45.1.91

649 Grotjan HE, Keel BA (1996) Data interpretation and quality control. In: Diamandis EP,
650 Christopoulos TK (eds) Immunoassay. Academic Press, San Diego, pp 51-95

651 Gulland FM (1999) Domoic acid toxicity in California sea lions stranded along the central
652 California Coast, May-October 1998. NOAA Tech. Memo. NMFS-OPR-8. USA National
653 Marine Fisheries Service, U.S. Department of Commerce

654 Gulland FM, Hall AJ, Greig DJ, Frame ER, Colegrove KM, Booth RK, Wasser SK, Scott-
655 Moncrieff JCR (2012) Evaluation of circulating eosinophil count and adrenal gland
656 function in California sea lions naturally exposed to domoic acid. J Am Vet Med
657 Assoc 241(7):943-949 https://doi.org/10.2460/javma.241.7.943

658 Hogg CJ, Rogers TL, Shorter A, Barton K, Miller PJO, Nowacek D (2009) Determination of
659 steroid hormones in whale blow: It is possible. Mar Mamm Sci 25:605-618
660 https://doi.org/10.1111/j.1748-7692.2008.00277.x

661 Hoffmeyer MS, Lindner MS, Carribero A, Fulco VK, Menéndez MC, Fernández Severini MD,
662 Diodato SL, Berasategui AA, Biancalana F, Berrier E (2010) Planktonic food and foraging
663 of *Eubalaena australis*, on Península Valdés (Argentina) nursery ground. Rev Biol Mar
664 Oceanogr 45.131-139

665 Hunt KE, Rolland RM, Kraus SD, Wasser SK (2006) Analysis of fecal glucocorticoids in the
666 North Atlantic right whale (*Eubalaena glacialis*). Gen Comp Endocrinol 148:260-272
667 https://doi.org/10.1016/j.ygcen.2006.03.012

668 Hunt KE, Moore MJ, Rolland RM, Kellar NM, Hall AJ, Kershaw J, Raverty SA, Davis CE,
669 Yeates LC, Fauquier DA, Rowles TK, Kraus SD (2013) Overcoming the challenges of
670 studying conservation physiology in large whales: a review of available methods. Conserv
671 Physiol 1(1):cot006 https://doi.org/10.1093/conphys/cot006

672 Hunt KE, Rolland RM, Kraus SD (2014) Detection of steroid and thyroid hormones via
673 immunoassay of North Atlantic right whale (*Eubalaena glacialis*) respiratory vapor. Mar
674 Mamm Sci 30:796-809 https://doi.org/10.1111/mms.12073

675 Hunt KE, Lysiak N, Moore M, Rolland RM (2017) Multi-year longitudinal profiles of cortisol
676 and corticosterone recovered from baleen of North Atlantic right whales (*Eubalaena*
677 *glacialis*). Gen Comp Endocrinol 254:50-59 https://doi.org/10.1016/j.ygcen.2017.09.009

678 Hunt KE, Robbins J, Buck CL, Bérubé M, Rolland RM (2019) Evaluation of fecal hormones
679 for noninvasive research on reproduction and stress in humpback whales (*Megaptera*
680 *novaeangliae*). Gen Comp Endocrinol 280:24-34
681 https://doi.org/10.1016/j.ygcen.2019.04.004

682 Hunt KE, Fernández Ajó A, Lowe C, Burgess EA, Buck CL (2021) A tale of two whales:
683 putting physiological tools to work for North Atlantic and southern right whales. In:

684 Madliger CL, Franklin CE, Love OP, Cooke SJ (eds) Conservation physiology. Oxford
685 University Press, Oxford, UK pp 205-226 doi:10.1093/oso/9780198843610.003.0012

686 Iverson F, Truelove J, Nera E, Tryphonas L, Campbell J, Lok E (1989) Domoic acid poisoning
687 and mussel-associated intoxication: preliminary investigations into the response of mice
688 and rats to toxic mussel extract. Food Chem Toxicol 27(6): 377-384

689 IWC (International Whaling Commission) (2001) Report of the workshop on the
690 comprehensive assessment of right whales: a worldwide comparison. J Cetacean Res
691 Manag (Spec Issue) 2:1-60

692 IWC (International Whaling Commission) (2011) Report of the southern right whale die-off
693 workshop. J Cetacean Res Manage (Suppl) 12:367-398

694 IWC (International Whaling Commission) (2015) Report of the second workshop on mortality
695 of southern right whales (*Eubalaena australis*) at Península Valdés, Argentina, 5–6 August
696 2014, Centro Nacional Patagónico, Puerto Madryn, Argentina. Document SC/66a/Rep/8

697 Johnson MP, Kelly G, Chamberlain M (2001) Changes in rat serum corticosterone after
698 treatment with metabotropic glutamate receptor agonists or antagonists J Neuroendocrinol
699 13:670-677 https://doi.org/10.1046/j.1365-2826.2001.00678.x

700 Keay JM, Singh J, Gaunt MC, Kaur T (2006) Fecal glucocorticoids and their metabolites as
701 indicators of stress in various mammalian species: a literature review. J Zoo Wildl Med 37:
702 234-244 https://doi.org/10.1638/05-050.1

703 Kenagy G, Masman D, Sharbaugh S, Nagy K (1990) Energy expenditure during lactation in
704 relation to litter size in free-living golden-mantled ground squirrels. J Anim Ecol 59(1):73-
705 88 DOI:10.2307/5159

706 Kenney RD (2009) Right whales *Eubalaena glacialis*, *E. japonica*, and *E. australis*. In: Perrin
707 WF, Würsig B, Thewissen JGM (eds) Encyclopedia of marine mammals. Academic Press,
708 London pp 962-972

709 Kraus SD, Hatch JJ (2001) Mating strategies in the North Atlantic right whale (*Eubalaena*
710 *glacialis*). J Cetacean Res Manage (Special Issue) 2:237-244

711 Lefebvre KA, Powell CL, Busman M, Doucette GJ, Moeller PDR, Sliver JB, Miller PE, Hughes
712 MP, Singaram S, Silver MW, Tjeerdema RS (1999) Detection of domoic acid in northern
713 anchovies and California sea lions associated with an unusual mortality event. Nat Toxins
714 7(3):85-92 https://doi.org/10.1002/(SICI)1522-7189(199905/06)7:3%3C85::AID-
715 NT39%3E3.0.CO;2-Q

716 Lemos LS, Olsen A, Smith A, Chandler TE, Larson S, Hunt K, Torres LG (2020) Assessment
717 of fecal steroid and thyroid hormone metabolites in eastern North Pacific gray whales.
718 Conserv Physiol 8. doi:10.1093/conphys/coaa110

719 Lightman SL (1992) Alterations in hypothalamic- pituitary responsiveness during lactation.
720 Ann N Y Acad Sci 652(1):340-346 https://doi.org/10.1111/j.1749-6632.1992.tb34365.x

721 Marón CF, Rowntree VJ, Sironi M, Uhart M, Payne RS, Adler FR, Seger J (2015a) Estimating
722 population consequences of increased calf mortality in the southern right whales off
723 Argentina. International Whaling Commission, Rep SC/66a/BRG/1 Cambridge

724 Marón CF, Beltramino L, Di Martino M, Chirife A, Seger J, Uhart M, Sironi M, Rowntree VJ,
725 (2015b) Increased wounding of southern right whale (*Eubalaena australis*) calves by Kelp
726 Gulls (*Larus dominicanus*) at Península Valdés, Argentina. PLOS ONE 10(11): e0142969
727 https://doi.org/10.1371/journal.pone.0142969

728 Marón CF, Lábaque MC, Beltramino L, Di Martino M, Alzugaray L, Ricciardi M, Fernández
729 Ajó AA, Adler FR, Seger J, Sironi M, Rowntree VJ, Uhart MM (2021) Patterns of blubber

730 fat deposition and evaluation of body condition in growing southern right whale calves
731 (*Eubalaena australis*). Mar Mamm Sci mms.12818. https://doi.org/10.1111/mms.12818

732 Masó M, Garcés E (2006) Harmful microalgae blooms (HAB); problematic and conditions that
733 induce them. Mar Pollut Bull 53:620-630 https://doi.org/10.1016/j.marpolbul.2006.08.006

734 Maucher JM, Ramsdell JS (2007) Maternal-fetal transfer of domoic acid in rats at two
735 gestational time points. Environ. Health Perspect 115:1743-1746

736 McAloose D, Rago MV, Di Martino M, Chirife A, Olson SH, Beltramino L, Pozzi LM,
737 Musmeci L, La Sala L, Mohamed N, Sala JE, Bandieri L, Andrejuk J, Tomaszewicz A,
738 Seimon T, Sironi M, Samartino LE, Rowntree V, Uhart MM (2016) Postmortem findings
739 in southern right whales *Eubalaena australis* at Península Valdés, Argentina, 2003-2012.
740 Dis. Aquat Organ 119:17-36. https://doi.org/10.3354/dao02986

741 Meylan S, Haussy C, Voituron Y (2010) Physiological actions of corticosterone and its
742 modulation by an immune challenge in reptiles. Gen Comp Endocrinol 169:158-166
743 https://doi.org/10.1016/j.ygcen.2010.08.002

744 McEwen BS, Wingfield JC (2003) The concept of allostasis in biology and biomedicine. Horm
745 Behav 43:2-15 https://doi.org/10.1016/S0018-506X(02)00024-7

746 Millspaugh, JJ, Washburn BE (2004) Use of fecal glucocorticoid metabolite measures in
747 conservation biology research: Considerations for application and interpretation. Gen
748 Comp Endocrinol 138(3):189-199 https://doi.org/10.1016/j.ygcen.2004.07.002

749 Moore SK, Trainer VL, Mantua NJ, Parker MS, Laws EA, Backer LC, Fleming LE (2008)
750 Impacts of climate variability and future climate change on harmful algal blooms and
751 human health. Environ Health 7, S4 https://doi.org/10.1186/1476-069X-7-S2-S4

752 Newman AEM, Chin EH, Schmidt KL, Bond L, Wynne-Edwards KE, Soma KK (2008)
753 Analysis of steroids in songbird plasma and brain by coupling solid phase extraction to

754 radioimmunoassay. Gen Comp Endocrinol 155(3):503-510
755 https://doi.org/10.1016/j.ygcen.2007.08.007

756 Palme R, Touma C, Arias N, Dominchin MF, Lepschy M (2013) Steroid extraction: get the best
757 out of faecal samples. Wien Tierarztl Monatsschr 100(9-10):238-46

758 Payne R, Brazier O, Dorsey EM, Perkins JS, Rowntree VJ, Titus A (1983) External features in
759 southern right whales (*Eubalaena australis*) and their use in identifying individuals. In:
760 Payne R (ed) Communication and Behavior of Whales. AAAs Selected Symposia Series
761 76. Westview Press, Colorado, pp 371-445

762 Perl TM, Bedard L, Kosatsky T, Hockin JC, Todd EC, Remis RC (1990) An outbreak of toxic
763 encephalopathy caused by eating mussels contaminated with domoic acid. N Engl J Med
764 322:1775-1780 doi: 10.1056/NEJM199006213222504

765 Pulido OM (2008) Domoic acid toxicologic pathology: a review. Marine Drugs, 6(2):180-219
766 https://doi.org/10.3390/md6020180

767 Rich EL, Romero LM (2005) Exposure to chronic stress downregulates corticosterone
768 responses to acute stressors. Am J Physiol Regul Integr Comp Physiol 288(6):R1628-
769 R1636 doi: 10.1152/ajpregu.00484.2004

770 Rolland RM, Hunt KE, Kraus SD, Wasser SK (2005) Assessing reproductive status of right
771 whales (*Eubalaena glacialis*) using fecal hormone metabolites. Gen Comp Endocrinol 142:
772 308-317 https://doi.org/10.1016/j.ygcen.2005.02.002

773 Rolland RM, Hunt KE, Doucette GJ, Rickard LG, Wasser SK (2007) The inner whale:
774 hormones, biotoxins and parasites. In: Kraus SD, Rolland RM (eds) The urban whale: North
775 Atlantic right whales at the crossroads. Cambridge, MA: Harvard University Press, pp.
776 232–272

777 Rolland RM, Parks SE, Hunt KE, Castellote M, Corkeron PJ, Nowacek DP, Wasser SK, Kraus
778 SD (2012) Evidence that ship noise increases stress in right whales. P Roy Soc B-Biol Sci
779 279(1737):2363-2368 https://doi.org/10.1098/rspb.2011.2429

780 Rolland RM, McLellan WA, Moore MJ, Harms CA, Burgess EA, Hunt KE (2017) Fecal
781 glucocorticoids and anthropogenic injury and mortality in North Atlantic right whales
782 *Eubalaena glacialis*. Endange Species Res 34:417-429 doi: 10.3354/esr00866

783 Romero LM, Dickens MJ, Cyr NE (2009) The reactive scope model a new model integrating
784 homeostasis, allostasis, and stress. Horm Behav 55:375-389
785 https://doi.org/10.1016/j.yhbeh.2008.12.009

786 Romero LM, Wingfield JC (2016) Tempests, poxes, predators, and people: stress in wild
787 animals and how they cope. Oxford University Press, New York, NY

788 Rowntree VJ, Payne RS, Schell DS (2001) Changing patterns of habitat use by southern right
789 whales (*Eubalaena australis*) on their nursery ground at Península Valdés, Argentina and
790 their long- range movements. J Cetacean Res Manag 2(Special Issue):133-143

791 Rowntree VJ, Valenzuela LO, Fraguas PF, Seger J (2008) Foraging behaviour of southern right
792 whales (*Eubalaena australis*) inferred from variation of carbon stable isotope ratios in their
793 baleen. International Whaling Commission Document SC/60/BRG23

794 Sapolsky RM, Romero LM, Munck AU (2000) How do glucocorticoids influence stress
795 response? Intergrating permissive, suppressive, stimulatory, and preparative actions.
796 Endocr Rev 21:55-89 https://doi.org/10.1210/edrv.21.1.0389

797 Sastre V, Santinelli N, Marino G, Solís M, Pujato L, Ferrario M (2007) First detection of
798 domoic acid produced by *Pseudo- nitzschia* species, Chubut coastal waters, Patagonia,
799 Argentina. Harmful Algae News 34:12-14

800 Scholin CA, Gulland F, Doucette GJ, Benson S, Busman M, Chavez FP, Cordaro J, DeLong R,
801 De Vogelaere A, Harvey J, Haulena M, Lefebvre K, Lipscomb T, Loscutoff S, Lowenstine
802 LJ, Marin R, Miller PE, McLellan WA, Moeller PD, Powell CL, Rowles T, Silvagni P,
803 Silver M, Spraker T, Trainer V, Van Dolah FM (2000) Mortality of sea lions along the
804 central California coast linked to a toxic diatom bloom. Nature 403:80-84
805 https://doi.org/10.1038/47481

806 Silvagni PA, Lowenstine LJ, Spraker T, Lipscomb TP, Gulland FM (2005) Pathology of
807 domoic acid toxicity in California sea lions (*Zalophus californianus*). Vet Pathol
808 42:184−191 https://doi.org/10.1354%2Fvp.42-2-184

809 Thomas PO, Taber SM (1984) Mother–infant interaction and behavioral development in
810 southern right whales, *Eubalaena australis*. Behaviour 88:42-60
811 https://doi.org/10.1163/156853984X00470

812 Tormosov D, Mikhaliev Y, Best P, Zemsky V, Sekiguchi K, Brownell RJ (1998) Soviet catches
813 of southern right whales *Eubalaena australis*, 1951-1971 Biological data and conservation
814 implications. Biol Conserv 86:185-197 https://doi.org/10.1016/S0006-3207(98)00008-1

815 Truelove J, Iverson F (1994) Serum domoic acid clearance and clinical observations in the
816 cynomolgus monkey and Sprague-Dawley rat following a single IV dose. Bull Environ
817 Contam Toxicol 52(4):479-486

818 Valenzuela LO, Sironi M, Rowntree VJ, Seger J (2009) Isotopic and genetic evidence for
819 culturally inherited site fidelity to feeding grounds in southern right whales (*Eubalaena*
820 *australis*). Mol Ecol 18:782-791 https://doi.org/10.1111/j.1365-294X.2008.04069.x

821 Valenzuela-Molina M, Atkinson S, Mashburn K, Gendron D, Brownell R (2018) Fecal steroid
822 hormones reveal reproductive state in female blue whales sampled in the Gulf of California,
823 Mexico. Gen Comp Endocrinol 261:127-135 https://doi.org/10.1016/j.ygcen.2018.02.015

824 Van Dolah FM (2000) Marine algal toxins: origins, health effects, and their increased
825 occurrence. Environ Health Perspect 108:133-141 https://doi.org/10.1289/ehp.00108s1133

826 Wasser SK, Hunt KE, Brown JL, Cooper K, Crockett CM, Bechert U, Millspaugh JJ, Larson
827 S, Monfort SL (2000) A generalized fecal glucocorticoid assay for use in a diverse array of
828 nondomestic mammalian and avian species. Gen Comp Endocrinol 120:260-275
829 https://doi.org/10.1006/gcen.2000.7557

830 Wells RS, Boness DJ, Rathbun GB, Rommel SA (1999) Behavior. In: Reynolds JE III (ed)
831 Biology of marine mammals. Smithsonian Institution Press, Washington, pp 324-422

832 Wilson C, Sastre AV, Hoffmeyer M, Rowntree VJ, Fire SE, Santinelli NH, Díaz Ovejero S,
833 D'Agostino VC, Maron C, Doucette G, Broadwater M, Wang Z, Montoya N, Seger J, Adler
834 F, Sironi M, Uhart M (2015) Southern right whale (*Eubalaena australis*) calf mortality at
835 Península Valdés, Argentina: Are harmful algal blooms to blame? Mar Mamm Sci 32:423-
836 451 https://doi.org/10.1111/mms.12263

837 Wittmaack C, Lahvis GP, Keith EO, Self- Sullivan C (2015) Diagnosing domoic acid toxicosis
838 in the California sea lion (*Zalophus californianus*) using behavioral criteria: A novel
839 approach. Zoo biology 34(4):314-320

840 Zabka TS, Goldstein T, Cross C, Mueller RW, Kreuder-Johnson C, Gill S, Gulland FMD (2009)
841 Characterization of a degenerative cardiomyopathy associated with domoic acid toxicity in
842 California sea lions (*Zalophus californianus*). Vet Pathol 46:105-119
843 https://doi.org/10.1354%2Fvp.46-1-105

844 Zerbini AN, Fernández Ajó AA, Andriolo A, Clapham PJ, Crespo EA, González R, Harris G,
845 Mendez M, Rosenbaum H, Sironi M, Sucunza F, Uhart M (2018) Satellite tracking of
846 Southern right whales (*Eubalaena australis*) from Golfo San Matías, Río Negro Province,

847 Argentina. Scientific Committee of the International Whaling Commission SC67b, Bled,
848 Slovenia.

849 **Table:**

850 **Table 1** Levels of domoic acid (DA), immunoreactive fecal corticosterone metabolites and
851 immunoreactive fecal cortisol metabolites in fecal samples of southern right whale from Golfo
852 Nuevo (GN), Argentina. Bahía Pirámide (BP); levels of DA below detection limit is indicated
853 with <dl; whales of unknown sex or age are reported as Unk; black stars indicate DA data
854 reported previously by D'Agostino et al. (2017); black dot refers to a sample collected from a
855 live lactating female sighted with two calves. Detection limit (S/N >3) ranges were determined
856 between 0.01 and 0.11 µg DA g^{-1} fecal sample [dry weight] and quantification limit (S/N >10)
857 ranges between 0.03 and 0.37 µg DA g^{-1} fecal sample [dry weight] in dependence of extracted
858 sample weight, respectively

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

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Figure legends:

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)

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 panels 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 panels C and D) was tested with 1:5 SPE extract; the best-fit regression equation is shown

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 right. Asterisks denote significant differences between groups, Welch T-test $p<0.05$. The black solid line indicates the mean for each group, and in parenthesis is the sample size for each group

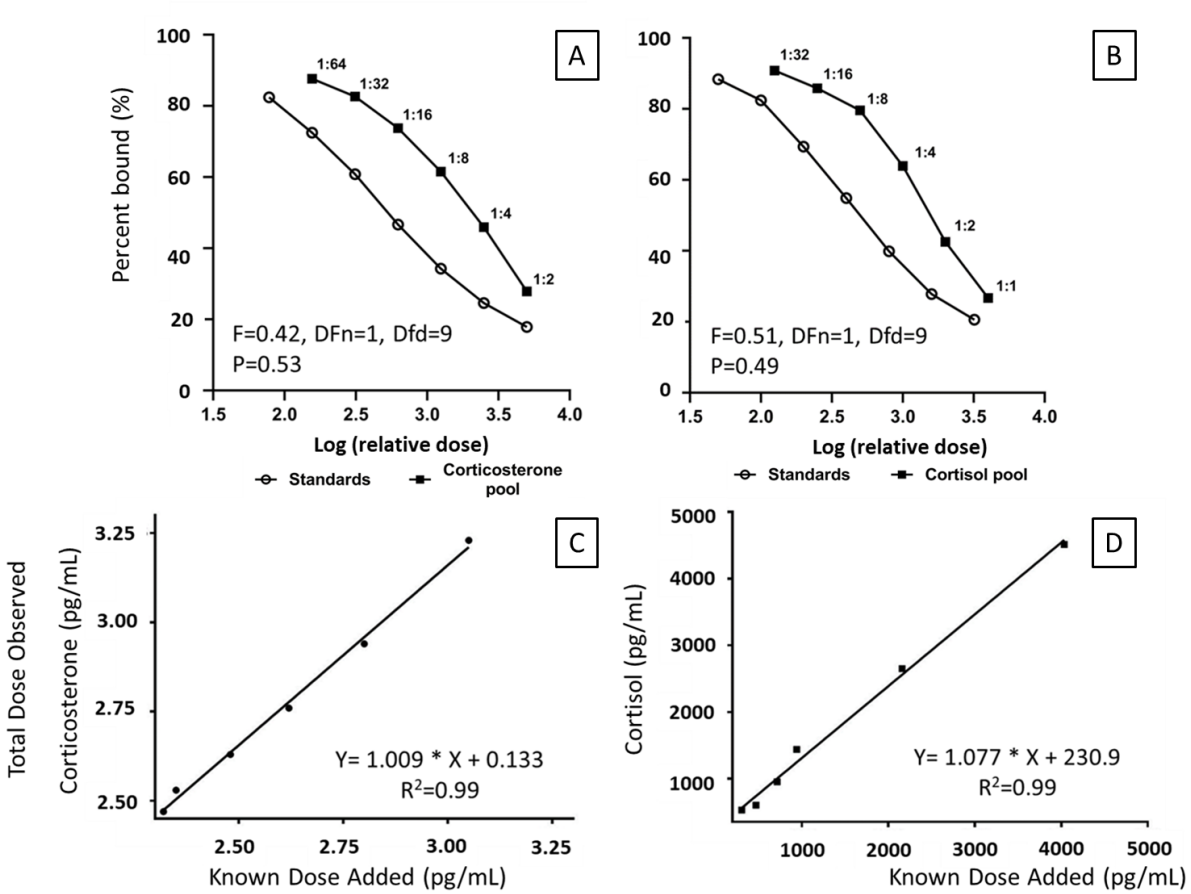
875 **Fig. 1**

876

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

880

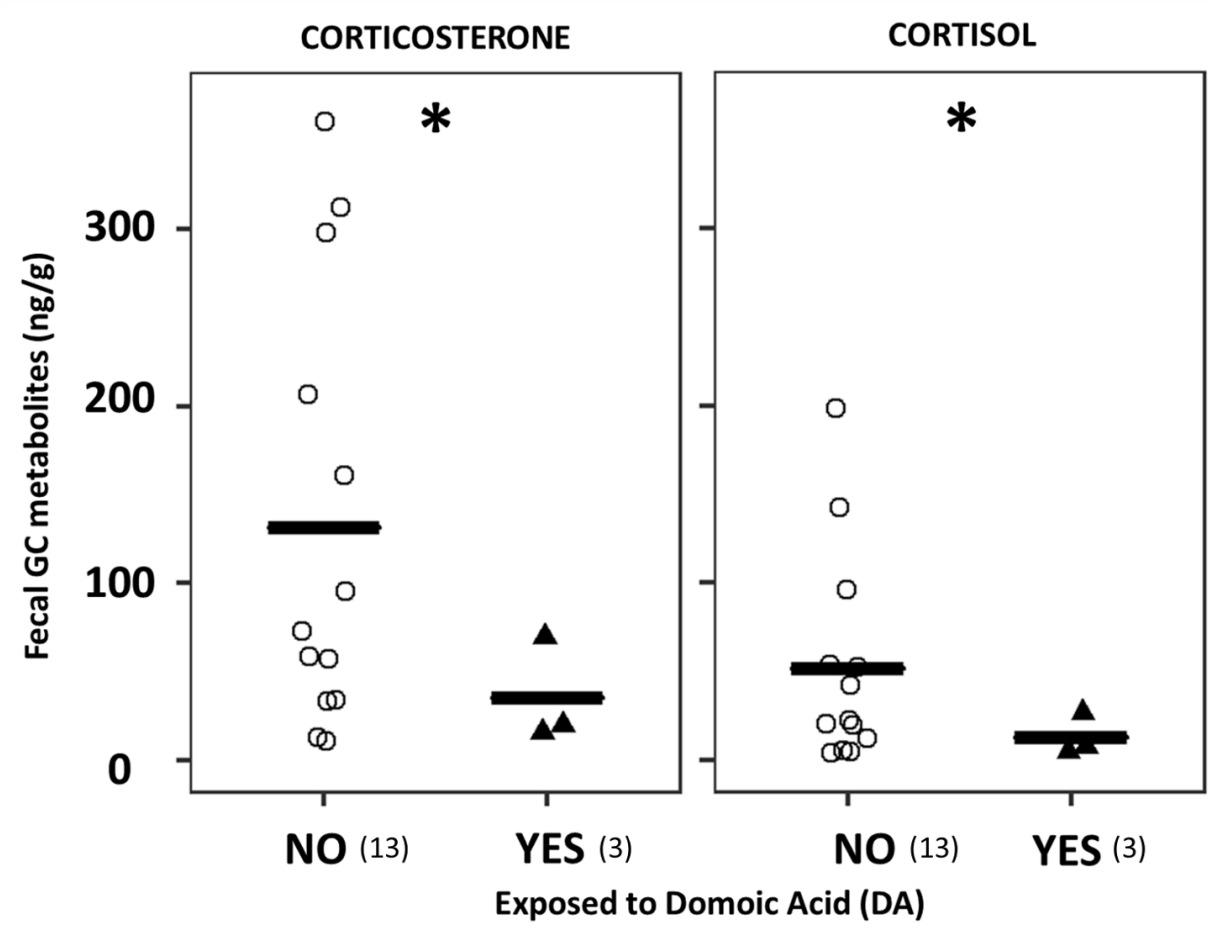
881 **Fig. 2**



882

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

889 **Fig. 3**



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