

Analysis of fecal glucocorticoids in the North Atlantic right whale (*Eubalaena glacialis*)

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Abstract

Very little is known about the endocrinology of the baleen whales. The highly endangered North Atlantic right whale (NARW; *Eubalaena glacialis*) is a good model species, because most NARW individuals are photo-identified with known histories. We used an ¹²⁵I corticosterone assay, shown to reliably measure cortisol metabolites, to determine glucocorticoid metabolite concentrations in 177 NARW fecal samples collected between 1999–2004 in the Bay of Fundy, Canada. Fecal glucocorticoid metabolite concentrations varied significantly with sex and reproductive category, being highest in pregnant females (mean \pm SE: 238.14 \pm 74.37 ng/g) and mature males (71.6 \pm 11.36), intermediate in lactating females (39.33 \pm 5.82), and lower in non-reproducing females (23.11 \pm 4.25) and immature males (34.33 \pm 5.01) and females (14.0 \pm 0.41). One case also suggests that glucocorticoids rise markedly in response to severe entanglement in fishing lines. Whales with fecal glucocorticoid content over 100 ng/g (termed “high-cort” samples) were rare, and included most pregnant females, some mature males, a fatally entangled whale, and several very young animals. Glucocorticoid concentrations were highly correlated with androgen concentrations in males and pregnant females. We analyzed the elution profiles of glucocorticoid and androgen metabolites in 13 samples with high-performance liquid chromatography (HPLC) to determine the extent to which androgen metabolites cross-react with our glucocorticoid assay. Males, pregnant females, non-pregnant females, and “high-cort” whales each had distinctly different immunoreactive HPLC profiles of glucocorticoid and androgen metabolites. A major glucocorticoid metabolite was prominent in all “high-cort” whales including the fatally entangled whale. The major fecal androgen was not testosterone but was instead a more nonpolar steroid (possibly dihydrotestosterone), which may be diagnostic of males. Androgen metabolites showed only minor cross-reactivity to our glucocorticoid assay, having a slight influence on glucocorticoid results in particular individuals. We conclude that fecal glucocorticoid analysis appears to be a useful measure of adrenal activity and reproductive condition for NARW.

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1. Introduction

The physiology of the baleen whales is arguably the most poorly understood of all the mammals. Their great size, aquatic habitat, and behavior prevent blood sampling as well as the most basic validations or experiments, and continuous observations are often limited to just a few minutes. As a result, fundamental endocrinology questions

remain unanswered for many baleen whales. Some limited progress has been made with samples from killed whales (reviewed in St. Aubin, 2001), but such samples are not from known individuals, are not useful for stress physiology (because they are confounded by the stress of pursuit and injury), and preclude repeated sampling. Development of new non-invasive methods is thus essential for understanding the endocrinology of most baleen whales and particularly for stress physiology. This study describes the validation and application of fecal glucocorticoid measures in the North Atlantic right whale (NARW; *Eubalaena glacialis*), one of the most endangered species of whale.

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Only about 350 NARW individuals remain (International Whaling Commission, 2001). Population size is small due to high mortality from ship strikes and entanglement in fishing gear (Knowlton and Kraus, 2001; Kraus et al., 2005), and a puzzling variability in female fecundity (Kraus et al., 2001; Reeves et al., 2001). The NARW is also notable due to its unusual mating system, involving prolonged sexual activity of large groups of whales at the surface (“surface active groups”, SAGs; Kraus and Hatch, 2001). Sperm competition appears to occur in this mating system, based on the NARW’s long penis and unusually large testes size. Based on the allometric relationship between testes and body mass in mammals, right whale testes are about six times larger than predicted (Brownell and Ralls, 1986). In right whales, the ratio of paired testes weight (maximum = 972 kg) to body weight (maximum estimated = 74,000 kg) is 1.3%, compared to 0.1% in fin whales (*Balaenoptera physalus*) and only 0.07% in the much larger blue whale (*Balaenoptera musculus*; from Table 1 in Brownell and Ralls, 1986).

The entire NARW population has been under intensive study for over 26 years, with photographic-identification of most individuals, many with known life histories (Hamilton and Martin, 1999; Kraus et al., 1986). The long-term dataset associated with the North Atlantic Right Whale Catalog thus offers an unsurpassed opportunity for linking physiological data with individual baleen whales of known history and reproductive status, and is an ideal species with which to test new non-invasive endocrinological methods for baleen whales.

We have recently shown that NARW feces float and can be collected, and that the androgen, progestin, and estrogen content of these samples reflects the age and reproductive state of the whales (Rolland et al., 2005). Here, we extend this technique to the fecal glucocorticoids as a potential

measure of adrenal activity and physiologic stress in free-swimming whales. To our knowledge this is the first time fecal glucocorticoid analysis has been applied to whales.

Fecal glucocorticoid analysis is now widely used for assessment of stress physiology in terrestrial wildlife. The glucocorticoids (e.g., corticosterone, cortisol) are secreted by the adrenal gland into the blood in response to a wide variety of environmental and social stressors. They are eventually excreted via bile into the gut, and end up as metabolites in feces (Wasser et al., 2000). Glucocorticoids may be elevated due to normal and predictable physiologic and environmental events, but, in general, elevated glucocorticoids provide an indicator that an adrenal stress response may be underway (Balm, 1999; Dallman and Bhatnagar, 2001). Provided fecal assays are well-validated and interpreted with care (Millspaugh and Washburn, 2004; Mostl and Palme, 2002; Wasser et al., 2000; Young et al., 2004), they have proven to have considerable practical utility in the field, correlating with a variety of known stressors in many mammalian taxa (Creel et al., 2002; Cavigelli et al., 2003; Foley et al., 2001; Goymann et al., 2001; Millspaugh et al., 2001; Rogovin et al., 2003; Sands and Creel, 2004; Wasser et al., 1997, 2000; Young et al., 2004).

However, fecal hormone assays always require careful validation. Fecal hormones are metabolized in the mammalian gut to a variety of different (and usually unidentified) metabolites, with unpredictable antibody affinities (Wasser et al., 1994, 2000). Most standard validations such as radiolabel infusions or adrenocorticotropic hormone (ACTH) injections are impossible in baleen whales. However, we can compare fecal glucocorticoid concentrations of whales we suspect, a priori, to have differing adrenal activity. Fecal glucocorticoids are known to increase during gestation (e.g., Foley et al., 2001), and should also be elevated in severely injured or entangled whales, and perhaps individu-

Table 1

NARW samples selected for HPLC analysis of immunoreactive glucocorticoids and androgens, shown with their original assay results for fecal glucocorticoids (Cort), fecal androgens (Andro), fecal estrogens (Estro), fecal progestins (Prog), androgen/glucocorticoid ratio (Andro/Cort), and androgen/estrogen ratio (Andro/Estro, reflecting gender)

		Cort	Andro	Estro	Prog	Andro/Cort	Andro/Estro
<i>“Normal” whales</i>							
02–03	Mature male	35	6880	83	332	197	83
04–12	Mature male	86	15,700	155	589	183	102
04–11	Immature male, 8 years	36	7800	140	424	216	56
04–13	Pregnant female (high-cort)	183	5320	12,100	109,400	29	0
04–31	Pregnant female (high-cort)	320	16,200	30,900	101,200	51	1
01–17	Pregnant female (high-cort)	643	16,300	23,800	245,100	25	1
02–19	Non-pregnant female	35	1860	260	244	53	7
04–29	Non-pregnant female (mildly entangled, good condition)	12	902	27	801	77	34
<i>Unusual “high-cort” whales</i>							
01–01	Fatally entangled male (very poor condition)	178	5120	249	225	29	21
01–02	“High-cort” calf, unknown sex	412	7520	339	231	18	22
02–10	“High-cort” calf, female	128	3440	679	263	27	5
04–32	“High-cort” non-pregnant female, unknown age	349	2060	283	970	6	7
02–15	“High-cort” non-pregnant female, yearling (~1.5 years)	826	266	12	177	0	23

Fecal hormone concentrations are in nanogram per gram. “Normal” whales are those with glucocorticoid concentrations typical for their reproductive category; note that pregnant females are normally “high-cort” whales (>100 ng/g). Unusual high-cort whales are those whales with glucocorticoids much higher than is typical for their reproductive category.

als involved in the intense social competition of the surface active groups. By contrast, fecal glucocorticoids should be relatively low in immature animals and non-reproductive individuals.

The high concentrations of fecal steroid hormones in some NARW (Rolland et al., 2005), presumably associated with their unusually large gonad size, suggests another validation issue that must also be addressed: cross-reactivity from excessively high concentrations of one hormone(s) elevating the apparent measurements of another hormone. Steroid hormones share the same basic structure, and thus assay antibodies bind not only to the target hormone, but also cross-react (bind more weakly) to almost all other steroid hormones. Hormones with cross-reactivities below ~1% are widely considered not to affect assay results. However, even a very low cross-reactivity can still cause major effects if there is a highly skewed ratio of the cross-reacting hormone to the target hormone. The extremely high gonadal steroid concentrations in NARW feces (Rolland et al., 2005) persuaded us to also assess the possible contribution of cross-reactivity of the reproductive steroids in the glucocorticoid assay.

The major objectives of this study were to: (1) identify a good glucocorticoid assay for use in NARW, by performing parallelism and accuracy validations for both a cortisol and a corticosterone assay; (2) explore whether fecal glucocorticoid concentrations vary significantly among whales of known age/sex class, reproductive status, or health status; (3) assess possible effects of assay cross-reactivity on glucocorticoid results, particularly by using HPLC to separate major immunoreactive androgen and glucocorticoid metabolites; and (4) ultimately, assess whether fecal glucocorticoid analyses shows promise as a measure of physiological stress in NARW.

2. Methods

2.1. Study population and sample collection

NARW fecal samples were collected in the Bay of Fundy, Canada, where large numbers of the population feed from July–September. Samples were either collected during on-going photo-identification shipboard surveys or by reliance on detection dogs (Wasser et al., 2004), trained to ride on the bow of a boat and alert its handler to floating fecal sample locations (Rolland et al., in press). Samples were scooped up from the water surface using a 300 µm nylon mesh dipnet (Sea-Gear Corp., Melbourne, FL) attached to a telescoping boathook. As much feces as possible was skimmed off the water surface, the saltwater drained off, and a 50–250 g subsample stored frozen (–20 °C) for later analyses. All samples were analyzed for hormone concentration within 9 months of collection. HPLC analyses also occurred within 9 months of collection for most samples.

We were able to assign many samples to known individual whales. Categories for age and reproductive state followed Hamilton et al. (1998); calf (less than 1-year-old); immature female (1–9 years old inclusive), “resting” female (female >9 years of age and not pregnant or lactating); lactating female; pregnant female (identified by sighting with a calf the following winter); immature male (1–9 years old inclusive); or mature male (over 9 years of age). The selection of 10 years as the age of sexual maturity is based on mean age of first calving of females. Whales can be classified as mature in three ways: (1) known age greater than 9 years; (2) whales of unknown age with at least an 8-year sighting history (because they were at least one year old at first sighting); and (3) or, for females, at least three

sightings with a calf. Based on estimated gestation length for southern hemisphere right whales (*Eubalaena australis*, Best, 1994) and calving date, samples from pregnant females in July–September probably represent months 7–9 of a 12–13 month gestation.

2.2. Hormone extraction and assay

Fecal samples were freeze-dried for 7 days, mixed, pulverized, sifted through a stainless steel colander, and mixed again. This process removes variation due to water content and equalizes hormone content throughout the sample (? Wasser et al., 1996). Dietary variation is not a major issue for this study, since NARW in the Bay of Fundy feed primarily on a single life stage of the copepod, *Calanus finmarchicus* (Murison and Gaskin, 1989). We extracted hormones using the methanol vortex method described in Wasser et al. (2000) and Hunt et al. (2004). Samples were diluted fourfold in assay buffer for the glucocorticoid assay. Extraction recoveries were not measured (see Hunt et al., 2004), but this method generally produces high and very consistent recoveries of tritiated steroids (Hunt et al., 2004; Khan et al., 2002).

We tested two glucocorticoid assays for parallelism, a 3H cortisol radioimmunoassay (RIA) developed in-house, and a ¹²⁵I corticosterone RIA (#02-120103; MP Biomedicals, Costa Mesa, CA). The cortisol assay failed the parallelism test, showing no antibody binding at any tested dilution (e.g., flat line near 100% bound; data not shown), and this assay was not investigated further. The corticosterone assay showed excellent parallelism (see Section 3) and was used for all further analyses. This assay is a double-antibody RIA that has been successfully validated for fecal glucocorticoid analyses in a wide variety of mammals and birds (e.g., Hunt et al., 2004; Wasser et al., 2000; Young et al., 2004). We used the manufacturer’s protocol except with half-volumes throughout; for assay details, see Wasser et al. (2000). All samples, standards and controls were assayed in duplicate, and non-specific binding tubes and blanks (0 ng/ml standard) in quadruplicate. To minimize effects of inter-extract variation and inter-assay variation, two subsamples were extracted from each fecal sample, each subsample was assayed in duplicate (in different assays), and the four results were averaged. We re-diluted and re-assayed any samples that (1) fell outside 15–85% on the standard curve, (2) had >7% coefficient of variation (CV) between duplicate tubes within one assay, or (3) had >20% CV between the two subsamples in different assays. In the rare event that assay controls fell outside the normal range, the entire assay was re-run. Intra-assay variation is 7.2%, inter-assay variation is 6.9%, and assay sensitivity is 0.2 ng/ml.

The antibody in this assay was raised in rabbit against corticosterone and has very low cross-reactivity to pure cortisol, but also binds well to a variety of fecal metabolites of both corticosterone and cortisol (Wasser et al., 2000). Known cross-reactivities are: desoxycorticosterone 0.34%, testosterone 0.25% (see Section 3), cortisol 0.05%, aldosterone 0.03%, progesterone 0.02%, androstenedione 0.01%, 5 α -dihydrotestosterone 0.01%; and <0.01% for 11-desoxycortisol, DHEA, DHEA-sulfate, 17 α - and β -estradiol, estriol, estrone, 17 α -hydroxyprogesterone, 20 α -dihydroprogesterone, pregnenolone, 17 α -hydroxypregnenolone, and cholesterol.

We express hormone results as nanograms of immunoreactive fecal hormone metabolite per gram of dried mixed feces. In this paper we refer generally to the parent hormones and their immunoreactive fecal metabolites as “glucocorticoids,” “androgens,” “progestins,” and “estrogens.” Androgen, progestin, and estrogen data from most of these same samples have been presented elsewhere (Rolland et al., 2005), and are discussed here only insofar as they relate to interpretation of glucocorticoid results.

2.3. Assay parallelism and accuracy

We tested assay parallelism using serial dilutions of two fecal samples from different whales (Diamandis and Christopoulos, 1996). Parallelism results were graphed as relative dose vs. percent-bound, and the slopes of the linear central portion of the curves were compared to the standard curve. Parallel slopes indicate that the antibody binds well to the fecal metabolites, across a range of concentrations (Diamandis and Christopoulos, 1996).

We tested accuracy of the corticosterone assay by spiking assay standards with an equal volume of NARW fecal pool diluted fourfold in assay

buffer, and assaying the spiked standards alongside the unspiked standards and the diluted fecal pool. Results were graphed as known standard dose (assay results from unspiked standards) vs. apparent dose (results of spiked standards minus results of the diluted fecal pool). A slope of 1.0 indicates that fecal components are not interfering with assay accuracy at the 1:8 dilution (Diamandis and Christopoulos, 1996).

2.4. Assay cross-reactivities

Because NARW feces contain highly skewed ratios of some hormones (Rolland et al., 2005), we tested cross-reactivities of four major steroids (progesterone, 17 β -estradiol, testosterone, and corticosterone) in all four corresponding fecal assays (progesterin, estrogen, androgen, and glucocorticoid). Twenty dilutions of known concentrations of each hormone were assayed four separate times (in separate assays) in each of the other hormone assays, and cross-reactivity at 50% bound was calculated. Note that we were not able to measure cross-reactivities for whale fecal metabolites of these four hormones—since those remain unknown—but only for the parent hormones that are likely to be the major hormones in blood. We then assessed whether these cross-reactivities were of practical significance for our NARW fecal samples, given their observed concentrations of immunoreactive steroids.

2.5. High-performance liquid chromatography

We used high-performance liquid chromatography (HPLC) to explore the nature of the immunoreactive glucocorticoids, and to further assess possible cross-reactivity from androgens. Our major questions were: (1) Are the major glucocorticoids corticosterone, cortisol, or some other glucocorticoid metabolite(s)? (2) Do any major androgen metabolites co-elute with glucocorticoid metabolites in a manner consistent with cross-reactivity? (3) Is the immunoreactive androgen(s) in NARW feces pure testosterone, or some other fecal androgen metabolite(s)?

2.6. Sample selection

For HPLC analyses, we selected samples to represent a variety of reproductive states and ages, as well as a range of glucocorticoid and androgen values (Table 1). The samples included eight “normal” individuals, (i.e., whales that had hormone profiles generally typical for their reproductive category), including three males, three pregnant females, and two resting females. We also analyzed five samples from whales with atypically high glucocorticoid concentrations for their reproductive category. These included a fatally entangled male, two calves, a female yearling, and a non-pregnant female of unknown age.

2.7. Sample filtration and purification for HPLC

From each sample, three subsamples of 0.4 g were extracted with 4.0 ml of 90% methanol, using the methanol vortex method (Hunt et al., 2004; Wasser et al., 2000). 3.0 ml of the resulting methanol supernatant from each subsample was dried, reconstituted in 1 ml 0.05 M citric acid (pH 4.0), combined with other subsamples, filtered through 0.2 μ m pore size, 25-mm diameter nylon syringe filters, loaded to a C-18 SPICE cartridge (Rainin Instruments) or Bond Elut Jr. cartridge (Varian Inc., Palo Alto, CA), washed with 0.05 M citric acid (pH 4.0) and distilled water, and eluted with 100% methanol. Elutions were dried again and reconstituted in 500 μ l methanol for HPLC. Finally, 20 μ l of tritiated progesterone (500 dpm/ μ l) was added to the extract to monitor inter-run differences in HPLC elution time. We controlled for variable recoveries by scaling the resulting HPLC profiles so that the area under the glucocorticoid curve (e.g., the total amount of immunoreactive glucocorticoids in all HPLC fractions) equals the original glucocorticoid assay result for that sample.

2.8. HPLC Gradient

For sample runs, 100 μ l of filtered concentrated NARW fecal extract was injected on to a reverse-phase C-18 column (Varian Instruments), and

120 1-ml fractions collected with a gradient solvent system (1 ml/min) as follows: 20–30% methanol (0–10 min), 30–40% (10–40 min), 40–50% (40–55 min), 50–80% (55–80 min), 80–100% (80–85 min), and 100% (85–120 min; Wasser et al., 2000). Elution times of several tritiated steroids (5 μ l of methanol containing \sim 5000 dpm of tritiated steroid) were measured three times each and are indicated on figures. Note that these times, as well as fraction numbers given throughout the text, indicate average elution times (e.g., #81 for testosterone); actual elution time may be 1 fraction (1 min) earlier or later in any given HPLC run (e.g., #80–82).

2.9. Assay of HPLC fractions

For glucocorticoid assay of HPLC fractions, we assayed quadruple the usual volume of sample (200 μ l of each HPLC fraction instead of the usual 50 μ l) to improve chances of detecting smaller immunoreactive peaks. Recoveries were corrected accordingly. We used usual assay volumes for androgen assays because the HPLC fractions generally had ample androgen content.

For the first six samples analyzed, we assayed all 120 fractions in singlicate in a single assay to assess what fractions were of interest. Fractions #1–20 and fractions #60–100 had many immunoreactive peaks in each of these six “high-cort” whales in both assays, while other fractions contained little immunoreactivity. Therefore, for all subsequent whales, we assayed only fractions #1–23 and #56–100, in a single assay in duplicate. We also re-assayed fractions #1–20 and #60–100 in singlicate for the original six whales, and averaged those results with those of the first assay. Two pregnant females had immunoreactive peaks whose tails extended into the “middle fractions” of #24–55, so, for those two whales only, fractions #24–55 were assayed (in duplicate) in a later assay for both hormones.

2.10. Statistical analyses

All hormone data were log-transformed before statistical analysis. Glucocorticoid data from whales of known sex and reproductive stage were compared with ANOVA, followed by post hoc tests with Fisher's PLSD. Calves, an extreme outlying sample from a yearling female (the only yearling in our study; see Section 3), and whales in highly unusual circumstances (e.g., fatally entangled whale; one NARW male found alone near Norway; post-mortem samples from whales killed by shipstrikes) were excluded from these analyses. Main effects and interactions of reproductive category, fecal androgens, fecal estrogens, and fecal progestins on fecal glucocorticoids were assessed with a generalized linear model (GLM). We also measured correlations of fecal glucocorticoids with fecal androgens, estrogens and progestins in various groups of whales. Since this correlation analysis involved twenty separate statistical comparisons, we used the Bonferroni correction to adjust the significance level to $\alpha = 0.05/20 = 0.0025$ for assessing significance of the correlation results. Assay parallelism and accuracy were assessed with Student's *t* tests for slope comparisons (Zar, 1999). All tests were done with Statview 5.0 for Macintosh.

3. Results

3.1. Sample collection and whale identifications

We collected and analyzed 177 NARW fecal samples from 1999–2004. Sixty-four samples could be assigned definitively to a particular individual, usually by visual confirmation of the whale defecating (while rolling at the surface or fluking), or by the sample's very close proximity to a lone NARW. These whales included 6 calves, 5 immature females (including a single yearling), 10 resting females, 12 lactating females, 3 pregnant females, 9 immature males, 7 mature males, 7 duplicate samples, and 6 samples from

whales in unusual circumstances (e.g., a fatally entangled adult male; an adult male found alone near Norway). In some cases these identifications have since been confirmed by fecal DNA analysis (R. Rolland, R. Bower, unpublished data). There were multiple samples from the same year for five females; these multiple samples generally had very similar results and data were averaged for analysis.

The remaining 113 samples were from unknown individuals. As discussed in Rolland et al. (2005), fecal samples from normal NARW can be accurately (100%) assigned to sex by androgen/estrogen ratio (AE ratio), and pregnant females are unambiguously identifiable (100%) by their high fecal progesterin concentrations. Mature males can also be identified with high accuracy by their rare combination of very high fecal androgens (over 12,000 ng/g) with relatively low progestins (below 2000 ng/g). Based on these hormonal profiles, we identified four samples from unknown whales likely to be pregnant females, and three from unknown whales likely to be mature males, and added these samples to our main glucocorticoid analysis. Thirty-three further samples were classed as males of unknown age; these were included in one analysis of androgen-glucocorticoid correlations. The remaining 73 samples were presumed to be from non-pregnant females of unknown age, or possibly from calves, and were not included in any statistical analyses.

3.2. Assay parallelism and accuracy

The corticosterone assay exhibited excellent parallelism, with slopes of diluted samples that were not significantly different from the slope of the standard curve ($t_6=1.708$ and $t_6=1.675$ for two different samples; critical value=2.447). The corticosterone assay also had good accuracy, with a slope not significantly different from 1 (slope of 0.96; $t_2=0.866$, critical value=4.303).

3.3. Fecal glucocorticoids and reproductive category

Healthy whales of differing sex and reproductive stage had significantly different concentrations of immunoreactive fecal glucocorticoids (Fig. 1; ANOVA, $F_{5,45}=13.678$; $p<0.0001$). Pregnant females had high fecal glucocorticoids (mean \pm SE = 238.14 ± 74.37), significantly higher than all other groups ($p<0.001$ for all comparisons). Lactating females had higher glucocorticoids (39.33 ± 5.82) than resting females (23.11 ± 4.25 ; $p=0.05$), and were not significantly different from either group of males. Females not actively reproducing had lower glucocorticoids than their corresponding age classes of males [immature females (14.0 ± 0.41) vs. immature males (34.33 ± 5.01), $p=0.03$; resting adult females vs. mature males (71.6 ± 11.36), $p=0.001$]. Within each sex, immature animals tended to have lower fecal glucocorticoids than mature animals, but these differences did not always reach significance (immature males vs. mature males, $p=0.05$; immature females vs. resting females, $p=0.30$; immature females vs. lactating females, $p=0.01$).

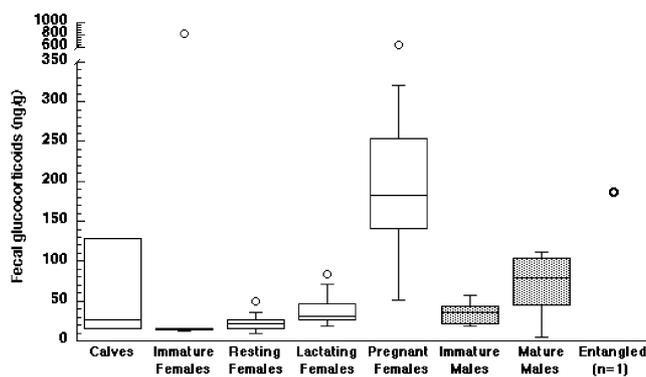


Fig. 1. Boxplots of fecal glucocorticoid concentrations of NARW of various ages and reproductive states. The line within the box is the median, the box encloses 25–75% of the data, the whiskers outside the box enclose 5–95% of the data, and circles are outliers. For comparison, the single circle on the far right is the sample from the fatally entangled whale. Note the y-axis break.

Calves were not included in the main ANOVA because most calves were of unknown sex; in addition, calves may ingest maternal hormones via milk fat. Both of these factors may contribute to high hormone variation in calves, and indeed calves had extremely variable concentrations of glucocorticoids and all other hormones. If calves are included in the ANOVA, they are not significantly different from any group except pregnant females. We also excluded data from the single yearling female. Her sample had the highest glucocorticoid content of any sample, two orders of magnitude higher than any other immature female; she may have been experiencing stress associated with post-weaning independent foraging or with participating in SAGs (she was in a SAG when the sample was collected). This sample was investigated further with HPLC (see below).

3.4. Correlations with other hormones

There was a highly significant association between glucocorticoid (GC) and androgen (A) concentrations across age-sex categories (“Category”). A General Linear Model (GLM) predicting glucocorticoid concentrations revealed a significant main effect of androgens ($p<0.0001$; $F=42.74$, $df=14$) as well as a significant A *Category interaction ($p<0.005$, $F=3.08$). This relationship between glucocorticoids and androgens is most apparent in male NARW, which exhibited a highly significant correlation of fecal androgens with fecal glucocorticoids (all probable males, $n=52$; $r=0.804$, $Z=7.772$, $p<0.0001$; males of known age only, $n=19$; $r=0.905$, $Z=6.000$, $p<0.0001$; Fig. 2). This correlation was strongest for mature males ($n=9$; $r=0.980$, $Z=6.099$, $p<0.0001$). A weaker association of these two hormones was also noted in immature males, although it did not reach statistical significance, set at 0.0025 after applying the Bonferroni correction ($n=9$; $r=0.748$, $Z=2.37$, $p=0.02$).

Interestingly, females of known age also had significantly correlated fecal androgens and fecal glucocorticoids

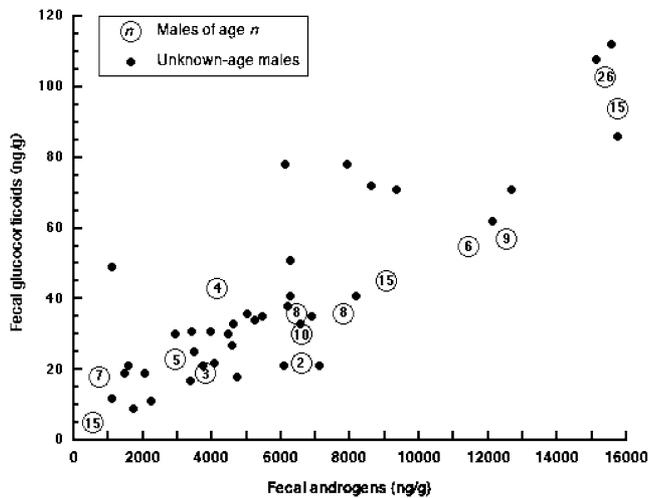


Fig. 2. Fecal glucocorticoid and androgen concentrations of male NARW. Identified males of known age are shown with open circles, with their age inside the circle. Males of unknown age are shown as black dots. Most males of unknown age are samples from unknown whales that were identified as males by androgen/estrogen ratio.

($n = 33$; $r = 0.823$, $Z = 6.384$, $p < 0.0001$). Female NARW can often have quite high androgen concentrations, and a few pregnant females had very high androgens in the same range as, or even slightly higher than, mature males (e.g., Table 1). The significant correlation in females overall was due mostly to the pregnant females, who had a significant relationship of glucocorticoids and androgens ($n = 7$; $r = 0.923$, $Z = 3.220$, $p = 0.0013$). Lactating females had a weaker, non-significant correlation ($n = 12$; $r = 0.593$, $Z = 2.045$, $p = 0.0408$) of glucocorticoids with androgens.

We suspected that the link between glucocorticoid and androgen correlation with age-sex category might be driven by a higher androgen/glucocorticoid ratio (A/GC) in some reproductive groups. We, therefore, included the A/GC ratio in the above analyses. Both A ($F = 54.54$; $p < 0.0001$) and the A/GC ratio ($F = 5.68$; $p < 0.025$) were significant predictors of GC, as was the interaction between Category and A/GC ratio ($F = 3.65$; $p < 0.012$) and the 3-way interaction between Category, A and A/GC ratio ($F = 3.18$; $p < 0.022$). Essentially, the higher the A/GC ratio, the higher the glucocorticoids. However, this effect is most pronounced when androgens are highest, as in adult males and some pregnant females, and is negligible in other groups.

We also explored relationships of glucocorticoids with fecal estrogens (E) and progestins (P). When added to the GLM above, E was highly significant ($p < 0.0001$, $F = 24.71$), as was the Category*E interaction ($p = 0.03$; $F = 2.34$) and the A*E interaction ($p < 0.0001$; $F = 39.74$). Progestins were also significant at predicting glucocorticoids in this model ($p = 0.005$, $F = 8.32$) as was the A*P interaction ($p = 0.003$, $F = 13.66$). These overall relationships may reflect generally high concentrations of all these hormones (E, P, and GC) in pregnant and lactating females. However, when these reproductive categories were examined individually, these hormonal correlations were not sta-

tistically significant. Glucocorticoids also did not show any statistically significant relationships with estrogens or progestins in any other group of whales (resting females, immature females, and both age classes of males).

3.5. “High-cort” whales

Most whales had glucocorticoid concentrations well below 100 ng/g. In our dataset of 177 NARW samples, only 16 samples were “high-cort” whales, defined as having fecal glucocorticoids above 100 ng/g. (All “high-cort” assay results were confirmed by analysis of additional fecal subsamples extracted and assayed independently.)

Six of seven pregnant females were high-cort whales. A sample from an eighth pregnant female that had been struck and killed by a ship also was a high-cort sample. (It is unknown whether she was killed instantly.) Three of ten mature males were high-cort whales, and several other mature males had glucocorticoids near 100 ng/g.

Two of the six calves in our study, along with the only yearling, had very high glucocorticoid concentrations—128 and 412 ng/g for the two calves, and 826 ng/g for the yearling, the latter two being the highest concentrations in our study. (The yearling female had extremely low androgen concentrations.) The other four calves all had glucocorticoids below 40 ng/g. As noted above, calves have remarkable variation in other hormone concentrations as well.

3.6. Entangled whales

We collected two samples from entangled whales, both of which could be assigned visual health assessment scores (Pettis et al., 2004). While anecdotal, these two rare samples offer a glimpse into the possible relationships between severity of entanglement, body condition, and fecal glucocorticoid concentrations.

The first was sample 01–01 from NARW #1102 (“Churchill”), an adult (minimum age 22 years) male who suffered a fatal entanglement in fishing line in 2001. A fecal sample was collected while research teams were approaching this whale for disentanglement efforts. At the time of sample collection, whale #1102 appeared emaciated, was covered in orange whale lice (*Cyamus ovalis*), and was assigned the poorest possible health assessment score. He vanished shortly after this sample was collected, and is presumed dead due to the severity of the entanglement, and because an attached satellite telemetry tag quit transmitting. This fecal sample is currently our most reliable example of a sample from a severely entangled, presumably stressed, and nutritionally compromised whale. The sample had glucocorticoids of 178 ng/g, the highest seen in our study for a mature male, and also had an androgen/estrogen ratio of 21 (Table 1), which is atypically low for a male. (This was the only male sample with an A/E ratio below 50).

The second sample was collected in 2004 from right whale #2320 (“Piper”), an adult female (minimum age 12 years) who, at that time, had been entangled in fishing line

for over two years. At the time of sample collection #2320 was behaving normally, appeared to be in good condition, and was assigned a good health assessment score. The fishing gear appeared loosely wrapped around her. About six months later #2320 was photographed again without the entanglement. Thus, this whale had apparently habituated to a mild entanglement that was not impeding normal feeding and other behaviors. This sample had very low glucocorticoid content (12 ng/g) and generally matched the hormonal profile of a normal resting female.

3.7. Assay cross-reactivities and potential relevance to NARW samples

For the glucocorticoid assay, we confirmed the manufacturer’s reported cross-reactivities for 17β-estradiol and progesterone. However, we measured a cross-reactivity of 0.25% for testosterone in the glucocorticoid assay, which is 2.5 times higher than the manufacturer’s reported value of 0.10%. The manufacturer subsequently replicated our results in their laboratory (MP Biomedicals technical support staff, personal communication, 2003).

After inspecting all hormone ratios for all NARW fecal samples and comparing them to the cross-reactivities, we identified two situations in which cross-reactivity is a possible concern: (1) Fecal progesterone of pregnant females may inflate glucocorticoid assay results. However, given the lack of correlation of progestins and glucocorticoids in pregnant females, this appears not to be a major problem for this study, and thus it seems likely that the pregnant females’ major fecal progestin(s) has lower cross-reactivity than pure progesterone. (2) Fecal androgens in all whales may inflate glucocorticoid results. Fecal androgens are indeed correlated with fecal glucocorticoids in NARW (see above). This correlation might be “biologically relevant” (e.g., NARW males with high androgens may experience increased reproductive competition and associated stress, given the degree of sperm competition suspected in this species) and/or it might be an artifact of androgen cross-reactivity.

Pure testosterone appears to have the highest cross-reactivity of all androgens that have been tested in this assay (see Section 2), and thus if NARW feces contain androgens other than testosterone, cross-reactivity may be less signifi-

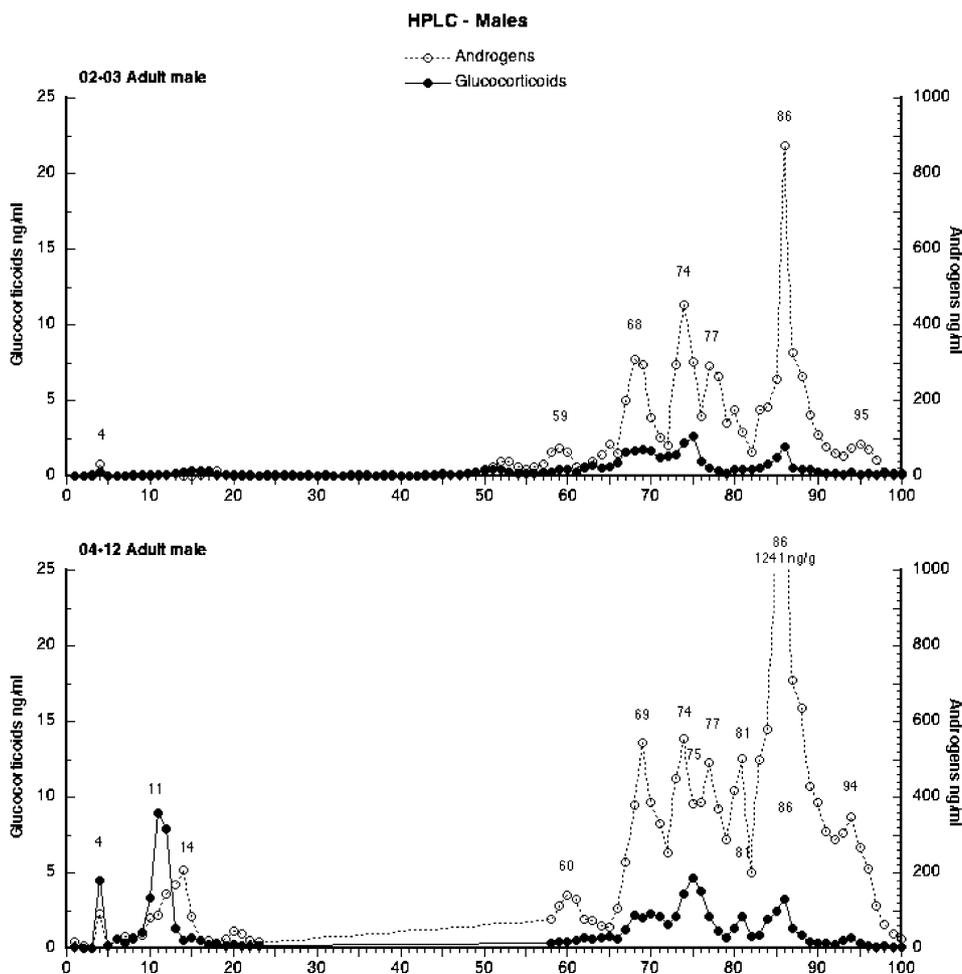


Fig. 3. HPLC profiles of immunoreactive glucocorticoids and androgens from fecal samples of two “normal” males—i.e., males with glucocorticoid results typical of their age and reproductive category. Elution times of major immunoreactive peaks are shown just above the peak. Elution times of pure tritiated steroids are shown with arrows at the bottom of Fig. 4.

cant than we had feared. We, accordingly, used HPLC to determine whether NARW feces contain significant amounts of pure testosterone, as well as the correspondence of all immunoreactive androgens with immunoreactive glucocorticoids in these samples.

3.8. HPLC analyses

HPLC analyses showed that metabolites cross-reacting to the corticosterone antibody, which we term “glucocorticoid-reactive metabolites”, tend to cluster in a polar section of fractions 4–15 and a non-polar section in fractions 60–95 (Figs. 3–8), as summarized in Table 2. Of those, the highest concentrations eluted in fractions 9–15 and 60–76. Pure corticosterone appears to be entirely absent, and pure cortisol, or some co-eluting glucocorticoid, is present only in some whales, particularly pregnant females. By contrast, androgen-reactive metabolites eluted most heavily in fractions 68–95. Testosterone (T) appears to be only a minor androgen and is often absent. Overlapping glucocorticoid and androgen fractions were primarily in fractions 76 and 86, and secondarily in fractions 68–71, and 81 (in adult males).

Table 2 illustrates that elution times of both glucocorticoid and androgen-reactive metabolites, and hence their overlap, varied markedly by sex, reproductive condition, and the apparent amount of stress they were experiencing. For example, a major glucocorticoid metabolite eluted in fraction 75 in all “unusual high-cort” whales (Figs. 4, 7, and 8) (but not in pregnant females, Fig. 5). Another major glucocorticoid metabolite eluted in fractions 61–65 primarily in pregnant females (Fig. 5) and the entangled adult male (Fig. 4). The fatally entangled male lacked the highly polar

glucocorticoid reactive peaks in fractions 9–15 seen in all other high glucocorticoid individuals (Figs. 5, 7, and 8).

A major androgen reactive metabolite eluted in fraction 85 (possible dihydrotestosterone, DHT) in all adult males (Fig. 3) except the fatally entangled adult male (Fig. 4), possibly indicating reproductive suppression in the latter individual. Pregnant females also exhibited androgen-reactive metabolites in fraction 85 (Fig. 5), as did one calf (Fig. 8, suspected to be a male for this reason).

Lastly, only the adult males had A/GC ratios >100 (all >180, Table 1). However, cross-reactivity between the androgen and glucocorticoid metabolites appeared to be minimal and limited to fractions 86 (putative DHT), and less so in fractions 75 and 81 (putative T; Fig. 3). The adult male with the highest androgen concentration also had the highest glucocorticoid concentration, but the glucocorticoid-reactive metabolites were mostly concentrated in fractions 4–12, where androgen-reactive metabolites were largely absent.

4. Discussion

The fecal glucocorticoid assay has great utility for discriminating among the different age, sex, and reproductive categories we examined for NARW. Despite the lack of radiolabel infusion or ACTH challenge studies in baleen whales, we tentatively conclude that this glucocorticoid assay does reflect adrenal activation in NARW. Based on reproductive class, fecal glucocorticoid concentrations were highest in pregnant females, followed by adult males (Fig. 1). Glucocorticoids are commonly elevated during mid- to late pregnancy in mammals (e.g., Foley et al., 2001). The relatively high glucocorticoid concentrations in adult males may result from intense reproductive competition associated with

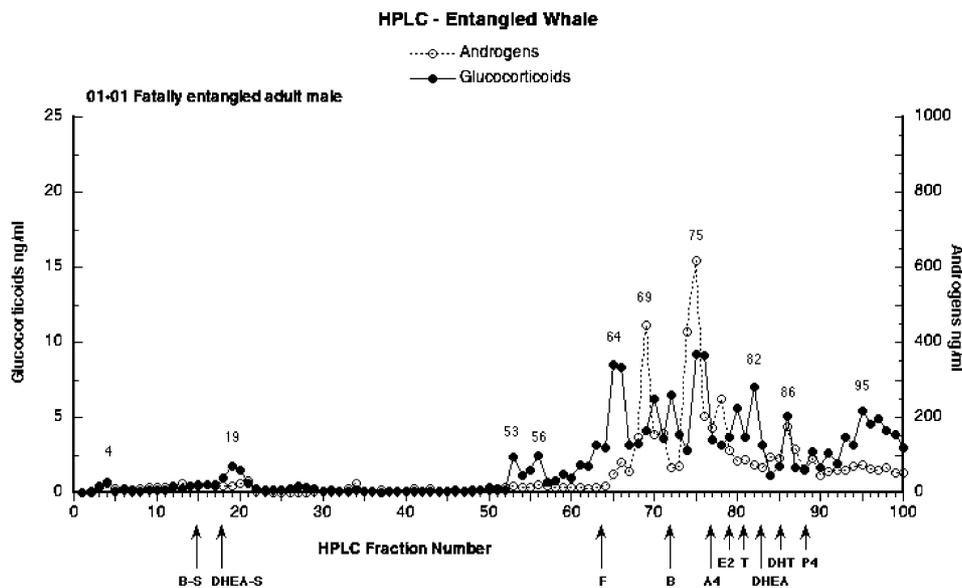


Fig. 4. HPLC profiles of immunoreactive glucocorticoids and androgens of a fecal sample from a fatally entangled mature male NARW who was in very poor body condition (see text). Elution times of major immunoreactive peaks are shown just above the peak. Elution times of pure tritiated steroids are shown at bottom with arrows: B-S, corticosterone sulfate; DHEA-S, dihydroepiandrosterone sulfate; F, cortisol; B, corticosterone; A4, androstenedione; E1, estrone; E2, 17 β -estradiol; T, testosterone; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; P4, progesterone.

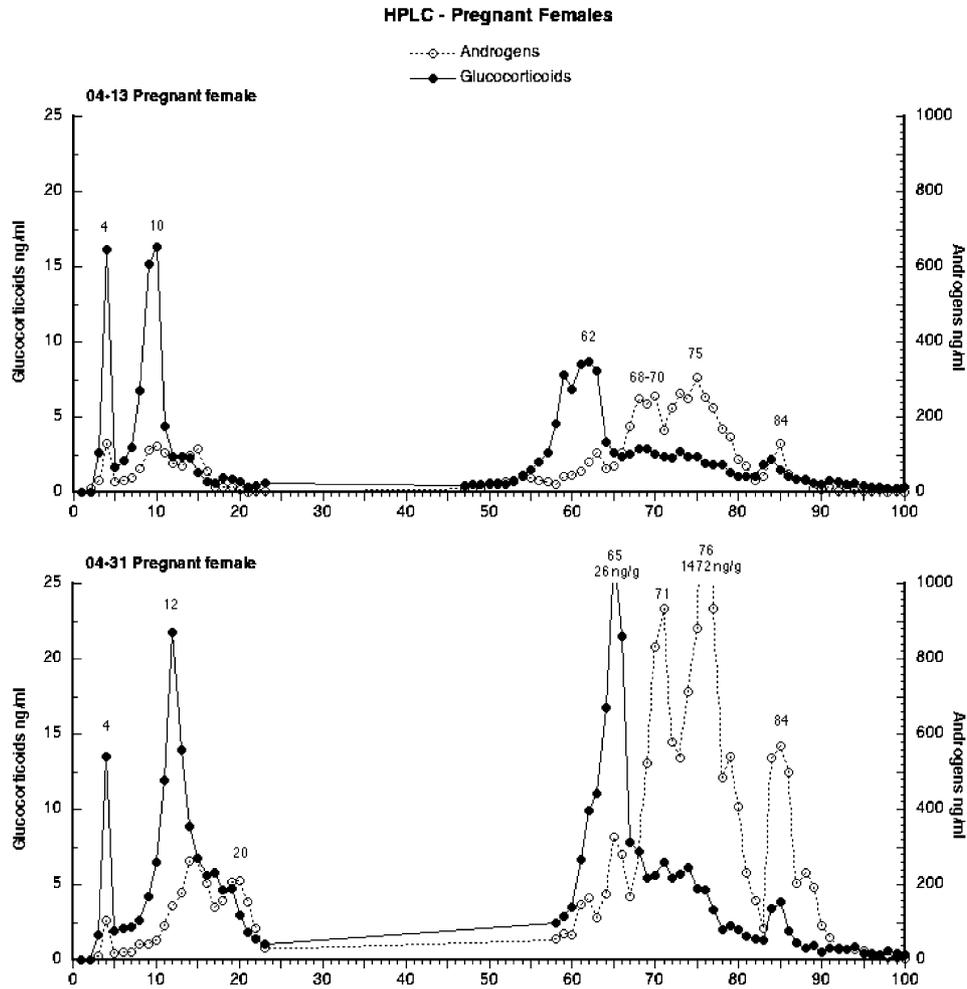


Fig. 5. HPLC profiles of immunoreactive glucocorticoids and androgens from fecal samples of two “normal” pregnant females—i.e., with glucocorticoid results typical of their age and reproductive category. These samples had “high-cort” glucocorticoid content >100 ng/g, as is typical of pregnant females. Elution times of major immunoreactive peaks are shown just above the peak. Elution times of pure tritiated steroids are shown with arrows at the bottom of Fig. 4.

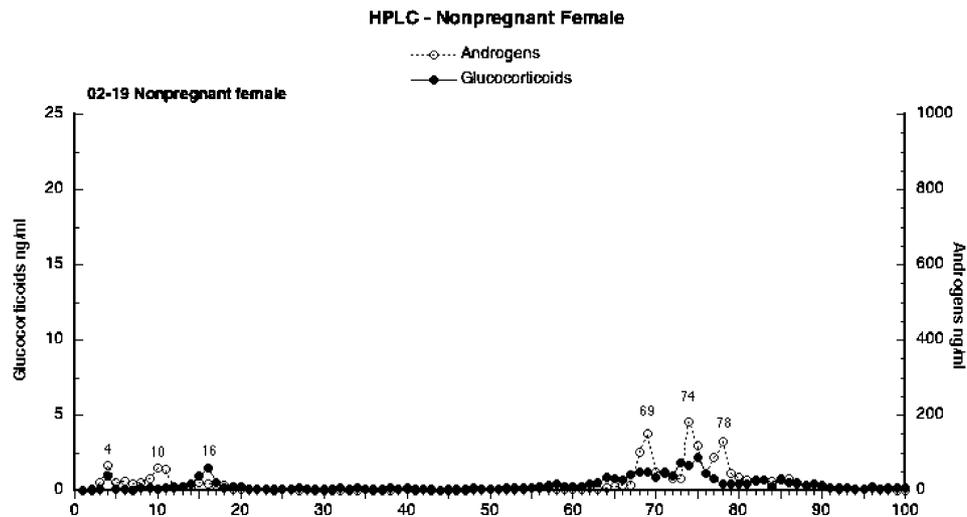


Fig. 6. HPLC profiles of immunoreactive glucocorticoids and androgens of a fecal sample from a “normal” non-pregnant female, i.e., with glucocorticoid results typical of their age and reproductive category. Elution times of major immunoreactive peaks are shown just above the peak. Elution times of pure tritiated steroids are shown with arrows at the bottom of Fig. 4.

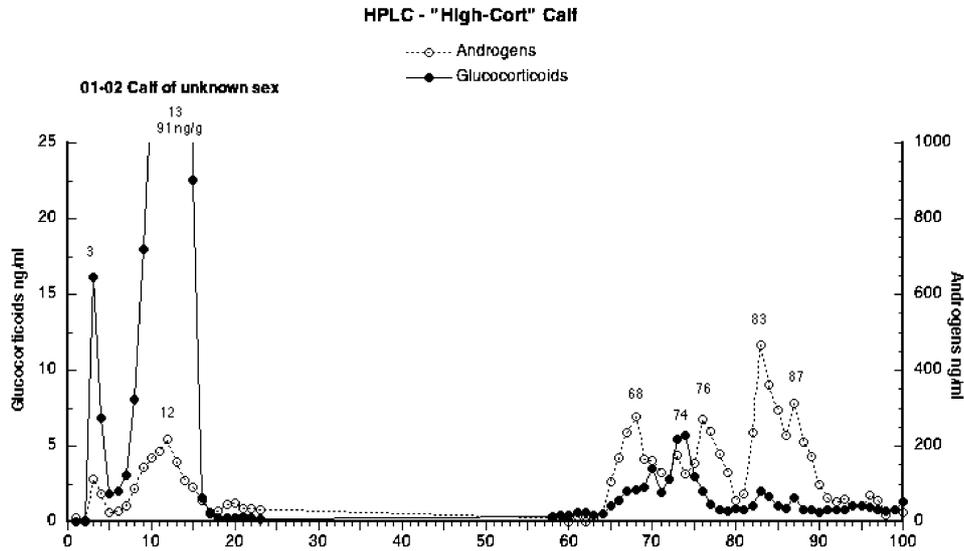


Fig. 7. HPLC profiles of immunoreactive glucocorticoids and androgens of a fecal sample from an unusually “high-cort” calf—i.e., whose glucocorticoid result was atypically high for calves. Elution times of major immunoreactive peaks are shown just above the peak. Elution times of pure tritiated steroids are shown with arrows at the bottom of Fig. 4.

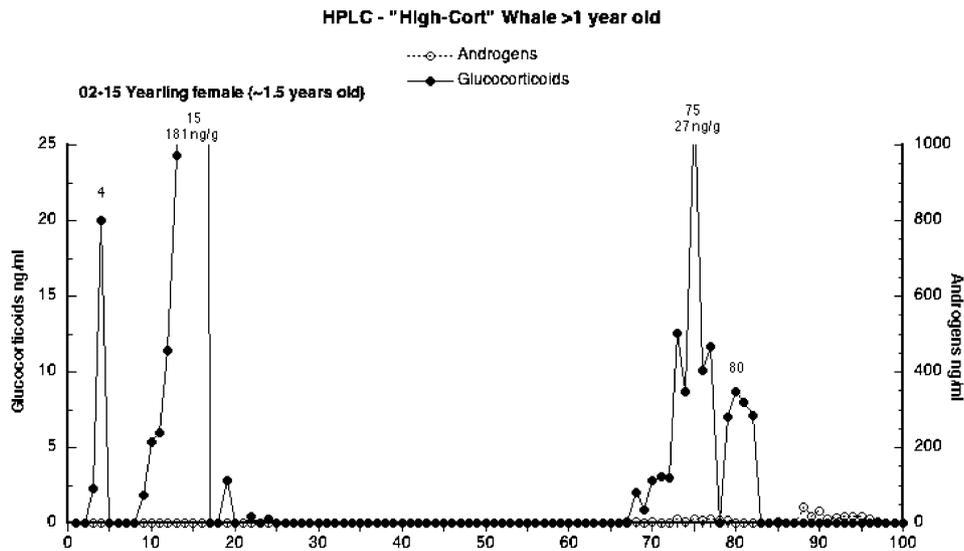


Fig. 8. HPLC profiles of immunoreactive glucocorticoids and androgens of a fecal sample from a NARW yearling with unusually “high-cort”. The yearling female had the highest fecal glucocorticoids of any whale in our study. Elution times of major immunoreactive peaks are shown just above the peak. Elution times of pure tritiated steroids are shown with arrows at the bottom of Fig. 4.

SAGs. However, some of the highest glucocorticoid concentrations among individual whales (aside from pregnant females) included a fatally entangled male in very poor health and three very young animals (two calves and a yearling). These young animals may have experienced stress associated with weaning, or from proximity to SAGs. It is also possible that variable high circulating concentrations of various hormones are a normal part of calf development in this species.

4.1. HPLC profiles and major immunoreactive metabolites

Although many different immunoreactive metabolites are present in different whales, HPLC profiles on the

whole tended to be consistent in whales of the same class (Table 2). Based on these profiles, we believe that the dominant glucocorticoid metabolites in NARW were found in HPLC fractions #9–15 (probably conjugates), 60–66 (with 65 possibly cortisol), and #73–76. Interestingly, the relative predominance of these peaks varied with reproductive condition and associated environmental stress (Table 2). The highly polar glucocorticoid-reactive metabolites (9–15) were present in all high-cort animals, except the fatally entangled male. The glucocorticoid-reactive peak #60–66 is very large in all pregnant females and also occurred in the fatally entangled whale. The glucocorticoid-reactive peak in #73–76 is the only one present in all high-cort whales, including the entan-

Table 2
HPLC fractions in which metabolites were detected by our glucocorticoid and androgen hormone assays as a function of the sex and reproductive condition of North Atlantic right whales

	Glucocorticoid fractions										
	4	9–15	19	60–66	68–72	73–76	80–82	84–86	95		
Adult males	+ ^a	+++ ^a				++	+	+			
Entangled adult male ^{b,g}			+	+++	++	+++	++	++	++	++	
Pregnant females	+++	+++		+++	++		++ ^c	++	++	++ ^c	
Non-Pg adult females	+ ^b	+			+						
Calves >1 year old ^g	+++	+++	+ ^d			+++	+++				
Calves <1 year old ^g	+++	+++			+	++					
	Androgen fractions										
	4	9–12	14–15	20	59–60	65	68–71	74–77	81	83–86	95
Adult males	+		+ ^a		+		++	+++	++	+++	++
Entangled adult male ^{b,f,g}							+++	+++			
Pregnant females	+	+	+	+		++	+++	+++		+++	
Non-Pg adult females	+ ^b	+ ^b					++	++			
Calves > 1 year old female ^g							++ ^b	++ ^b			
Calves < 1 year old (1m:1f) ^g	+	++ ^c	+ ^b				++	++		+++ ^c	

^a Only the highest androgen male.

^b Only one individual.

^c Only the highest A and GC pregnant female.

^d Only the highest GC calf.

^e Probably a male.

^f Appears to be reproductively suppressed; note lack of peaks between #81–95.

^g Unusual “high-cort” whales; see Table 1.

gled whale. Since peak #73–76 seems to correlate with the “high-cort” status of the whale, and appears to not be due to cross-reactivity, the presence and height of peak #75 may prove to be one of the most useful diagnostic tools for interpretation of glucocorticoid assay results in NARW.

The most significant androgen metabolite in NARW appears to be in fractions #83–86, and likely includes dihydrotestosterone (DHT, fraction #85). Interestingly, this peak is absent in the fatally entangled male (whose androgen concentrations were relatively low), implying that he may have been experiencing stress-related reproductive suppression. This peak was also present in the calf of unknown sex, perhaps indicating it was a male. It was additionally present in two out of three pregnant females, and may indicate that they were carrying male fetuses. Other notable androgen reactive peaks included #68–71 and #74–77, which occur to some degree in almost all whales.

4.2. Cross-reactivity

Our results suggest that cross-reactivity may be more problematic than currently appreciated, particularly when hormone ratios are highly skewed. Cross-reactivities below 1% are usually dismissed as insignificant. However, our study indicates that cross-reactivity can still influence hormone concentrations when the cross-reactive hormone is over 100 times more concentrated than is the hormone being measured. Such cross-reactivity problems could also result from unmeasured hormone metabolites (e.g., androgen metabolites undetected by the androgen assay but cross-reacting with the glucocorticoid assay).

Fortunately, there appears to be very little pure testosterone in NARW feces, and though testosterone is apparently causing some cross-reactivity (at fraction #81 in males), its contribution to overall glucocorticoid immunoreactivity appears quite minor. Cross-reactivity does appear to occur to some degree with the dominant fecal androgen around fraction #85 (possibly DHT) in some pregnant females and adult males. If a whale has a very high androgen/glucocorticoid ratio, then cross-reactivity at fraction #85 could have a significant impact on the overall glucocorticoid result.

Based on our HPLC results, we tentatively conclude that the testosterone-glucocorticoid correlation in NARW males is probably due to a combination of a “biologically relevant” correlation (e.g., courtship group competition) and a relatively small degree of cross-reactivity. However, larger sample sizes and more HPLC analyses will be needed to test this question thoroughly. We also recommend the use of gas chromatography-mass spectrometry to identify the major immunoreactive metabolites, and subsequently to experimentally measure their actual cross-reactivities to assay antibodies. Progesterin and estrogen HPLC profiles should be simultaneously investigated as well.

5. Conclusions

Fecal glucocorticoid analysis appears useful for identifying a variety of stressors and discriminating different reproductive and health categories of NARW. In addition, this study illustrates that HPLC can be a useful technique for interpreting fecal hormone results in baleen whales. Since other standard validations are not possible in whales,

HPLC offers a viable avenue for validating endocrine analyses in free-swimming whales, allowing investigation of the nature and identity of the fecal metabolites, identifying metabolites of greatest interest, and assessing possible cross-reactivity impacts.

Our study also illustrates the importance of having samples from known individuals. With the photographic identification database in the North Atlantic Whale Catalog and the associated long-term life history data, we were able to place our fecal samples into known age/sex categories. This enabled us to tie HPLC profiles to age/sex category, allowing the different HPLC profiles to fall into place; i.e., revealing that NARW in the same age/sex category had markedly similar HPLC profiles. Only in this way were we able to identify the probable dominant fecal androgen and glucocorticoid metabolites.

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