1   **Adrenal responses of large whales: integrating fecal aldosterone as a**
2   **complementary biomarker to glucocorticoids**
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4   Elizabeth A. Burgess[1]*, Kathleen E. Hunt[1,2], Scott D. Kraus[1] and Rosalind M. Rolland[1]

5   [1]*Anderson Cabot Center for Ocean Life, New England Aquarium, 1 Central Wharf,*
6   *Boston, MA 02110*

7   [2]*Present address: Center for Bioengineering Innovation, Northern Arizona University,*
8   *Flagstaff, AZ 86011*

9   *Corresponding author: eburgess@neaq.org*

10

11   **ABSTRACT**

12   Until now, physiological stress assessment of large whales has predominantly focused on

13   adrenal glucocorticoid (GC) measures. Elevated GC concentrations in feces (fGC) are

14   known to reflect stressful disturbances, such as fishing gear entanglement and human-

15   generated underwater noise, in North Atlantic right whales (*Eubalaena glacialis*).

16   However, there can be considerable variation in GC production as a function of sex and

17   life history stage, which may confound the interpretation of fGC levels. Additionally, GC

18   antibodies used in immunoassays can cross-react with other fecal metabolites (i.e., non-

19   target steroids), potentially influencing fGC data. Here, aldosterone concentrations

20   (fALD; aldosterone and related metabolites) were measured in fecal samples from right

21   whales (total n = 315 samples), including samples from identified individuals of known

22   life history (n = 82 individual whales), to evaluate its utility as a complementary

23   biomarker to fGC for identifying adrenal activation. Concentrations of fALD were

24   positively correlated with fGCs in right whales ($r = 0.59$, $P < 0.001$), suggesting

25   concurrent secretion of these hormones by the adrenal gland. However, fALD levels were

less influenced by concentrations of reproductive steroids in feces, minimizing the potential confounder of assay cross-reactivity in samples with highly skewed hormone ratios. Across different life history states for right whales, fALD concentrations showed similar patterns to those reported for fGC, with higher levels in pregnant females (35.9 ± 7.6 ng/g) followed by reproductively mature males (9.5 ± 0.9 ng/g) ($P < 0.05$), providing further evidence of elevated adrenal activation in these groups of whales. The addition of fALD measurement as a biomarker of adrenal activation may help distinguish between intrinsic and external causes of stress hormone elevations in large whales, as well as other free-living wildlife species, providing a more comprehensive approach for associating adrenal activation with specific natural and anthropogenic stressors.

1.0 INTRODUCTION

Marine mammals are increasingly exposed to complex and myriad threats, involving anthropogenic disturbances (e.g., underwater noise, shipping traffic, fishing activities, pollution, climate change) and environmental challenges (e.g., prey availability, disease, extreme weather events) (Davidson et al., 2012; Schipper et al., 2008). Such threats are often indirect and cumulative (Halpern et al., 2008; Maxwell et al., 2013), which further complicates the ability to detect and monitor impacts on marine mammal populations. Nonetheless, effective conservation and management require clarity on the causes of

49 disturbance and the consequences for vital rates and population dynamics of marine

50 mammals (Fleishman et al., 2016).

51

52 To investigate the impact of disturbance on wild animals, many researchers have focused

53 on endocrine mechanisms by measuring glucocorticoid (GC) hormones (reviewed by

54 Busch and Hayward, 2009; Cooke and O'Connor, 2010), which have become the most

55 widely used biomarkers of stress responses in wildlife species (Millspaugh and

56 Washburn, 2004; Sheriff et al., 2011). When an animal is confronted with a stressor,

57 higher brain areas initiate physiological responses that involve both the sympathetic

58 nervous system and the hypothalamic-pituitary-adrenal (HPA) axis. As part of this stress

59 response, the adrenal gland secretes GC hormones (mainly cortisol or corticosterone) into

60 the bloodstream (Romero and Wingfield, 2016). Glucocorticoids mobilize the energy

61 needed to cope with and respond adaptively to challenges by stimulating release of

62 glucose, fatty acids and triglycerides from storage sites to exercising muscles and the

63 brain (Romero and Wingfield, 2016). Consequently, circulating GC concentrations above

64 baseline levels are symptomatic of adrenal activation (Romero and Wingfield, 2016), and

65 these measureable changes in GC can provide quantitative information about whether

66 anthropogenic disturbance and environmental challenges are affecting individuals

67 (reviewed by Atkinson et al., 2015; Busch and Hayward, 2009; Cooke and O'Connor,

68 2010).

69

70 The development of techniques to measure hormones in noninvasive sample types (as

71 alternatives to blood) has enabled physiological study of wildlife species that are difficult

72 to sample and/or capture (Amaral et al., 2010; Atkinson et al., 2015; Hunt et. al., 2013;

73 Keay et al., 2006; Touma and Palme, 2005; Wasser et al., 2010). For example, analysis of

74 GC metabolites in feces (fGC) has provided a means of assessing stress responses in the

75 largest terrestrial mammal (African elephants, *Loxodonta africana*; Ahlering et al., 2013;

76 Foley et al., 2001) and free-swimming marine mammals (e.g., killer whales, *Orcinus*

77 *orca*; Ayres et al., 2012; dugongs, *Dugong dugon*; Burgess et al., 2013), including a large

78 whale species of body size up to 17 m and 40-50 tons, the North Atlantic right whale

79 (*Eubalaena glacialis*) (Hunt et al., 2006; Rolland et al., 2005). North Atlantic right

80 whales (hereafter, right whales) remain one of the most endangered whale species

81 (Thomas et al., 2015), in part due to anthropogenic impacts encountered during their

82 migration along a densely human-populated coastline from Florida to the Gulf of St.

83 Lawrence (Kraus et al., 2016). Fecal GC concentrations in right whales, in conjunction

84 with acoustic and ship traffic data, provided evidence that increasing underwater noise

85 from large vessels is a chronic stressor for these whales (Rolland et al., 2012). Highly

86 elevated fGC levels have also been reported for right whales chronically entangled in

87 fishing gear, reflecting the extreme adrenal response experienced by whales suffering

88 such physically stressful circumstances (Hunt et al., 2006; Rolland et al., in prep).

89

90 When interpreting fGC data, it is important to understand that baseline GC production

91 can be influenced by intrinsic biological factors, such as sex and reproductive stage of

92 individual animals (Boonstra, 2005; Goymann, 2012; Millspaugh and Washburn, 2004;

93 Wingfield, 2005). In right whales, elevated fGC concentrations have been reported for

94 pregnant females and reproductively mature males (Hunt et al., 2006). However,

distinguishing significant variation in fGC associated with life history can be challenging when the metabolic pathways of hormone excretion are not fully understood (Goymann, 2012; Millspaugh and Washburn, 2004; Touma and Palme, 2005). A major consideration for quantifying and interpreting GCs in feces is that circulating parent hormones are extensively modified and degraded into various metabolites for elimination from the body (Goymann, 2012; Touma and Palme, 2005). Any sex-specific differences in hormone metabolism could influence fGC measures because antibodies used to quantify GC concentration in immunoassays typically have affinity to an array of structurally similar steroids, and could bind to non-target (functionally different) hormone metabolites present in the sample, i.e., cross-reactivity (Goymann, 2012; Krasowski et al., 2014; Touma and Palme, 2005). For right whales, some individuals have gonadal steroids in extremely high concentrations in their feces (Rolland et al., 2005), such that even cross-reactivity reportedly as low as 1% could have appreciable effects on apparent fGC results (Hunt et al., 2006). Clarifying this issue is troublesome for studies on large whales, because conventional methods to verify routes of hormone metabolism and excretion, and yield information on the proportion and structure of resulting fecal metabolites are impossible to perform (e.g., radiometabolism studies involving injection of radiolabeled hormone into the animal) (see Hunt et al., 2015; 2013). For studies that depend on fecal-based hormone assessments of wild populations, caution is warranted when relying solely on fGC as an indicator of stress responses (reviewed by Dantzer et al., 2014), and development of supplemental biomarkers of adrenal function is desirable to enhance the specificity of fGC assessments.

118   Aldosterone is an additional steroid hormone that also functions in the HPA stress

119   response (Kubzansky and Adler, 2010). Aldosterone is a primary mineralocorticoid

120   secreted by the adrenal cortex, which acts on the kidneys to maintain electrolyte balance,

121   retain water, and stabilize blood pressure and blood volume in the mammalian body

122   (Kubzansky and Adler, 2010). Production of aldosterone is influenced by two main

123   hormonal systems, the HPA and the renin-angiotensin system, i.e., aldosterone is under

124   dual control. During times of low HPA axis activity (basal conditions), aldosterone

125   secretion is primarily controlled by renin-angiotensin system (Kubzansky and Adler,

126   2010). However, during a stress response, activation of the HPA axis also stimulates

127   aldosterone release via adrenocorticotrophic hormone (ACTH), the same hormone that

128   stimulates the secretion of GCs (Romero and Wingfield, 2016). The role of aldosterone in

129   the stress response appears to be stabilization of blood pressure and restoring

130   cardiovascular homeostasis in the face of alterations in ionic and osmotic balance that

131   occur due to increased catabolic activity (induced by elevated GCs).

132

133   Because of its role in osmoregulation to prevent loss of sodium and water, aldosterone is

134   especially interesting in fully marine mammals that live in a hyperosmotic (saline)

135   environment (Ortiz, 2001). Adrenocortical stimulation of bottlenose dolphins (*Tursiops*

136   *truncatus*) held in captivity produced extreme responses in aldosterone secretion, with

137   three-fold increases in circulating levels of aldosterone following exogenous ACTH

138   administration (Thomson and Geraci, 1986). In bottlenose dolphins and belugas

139   (*Delphinapterus leucas*), aldosterone release has been shown to rise in the blood

140   coincident with cortisol increases under situations of acute stress, such as encirclement,

141   capture and/or handling (Hart et al., 2015; Ortiz and Worthy, 2000; Schmitt et al., 2010;

142   St. Aubin and Geraci, 1989; St. Aubin et al., 1996; Thomson and Geraci, 1986), as well

143   as experimental exposure to cold water (Houser et al., 2011) or loud sound (Romano et

144   al., 2004). The prominence of aldosterone in the stress response suggests an important

145   need for sodium reabsorption to enhance water conservation and secure electrolyte

146   balance during challenging times, which could be vital for an animal that could be

147   deprived (at least in the short term) of dietary water. Aldosterone appears to have a key

148   function in the stress response of smaller cetaceans studied to date, and may serve to be a

149   highly useful and more sensitive indicator of stress in marine mammals (reviewed by

150   Atkinson et al., 2015; Fair and Becker, 2000; Ortiz, 2001; St Aubin and Dierauf, 2001).

151

152   In this study, we investigated fecal aldosterone (fALD; aldosterone and its

153   immunoreactive metabolites) of free-swimming North Atlantic right whales as a

154   complementary biomarker to GCs for reliably assessing adrenal activation, using

155   noninvasive methods. Our objectives were to: (1) validate an immunoassay to measure

156   fALD in right whales; (2) assess the relationship between fALD and fGC concentration;

157   (3) investigate the influence of reproductive hormone concentrations on immunoreactive

158   fALD and fGC results; (4) determine the variation in fALD concentrations among whales

159   of known sex and reproductive state; and ultimately, (5) evaluate the utility of fALD to

160   enhance the specificity of noninvasive fecal hormones to detect stress responses in free-

161   living large whales.

162

163   **2.0 METHODS**

7

2.1 Sample collection

A total of 315 fecal samples were collected from North Atlantic right whales in feeding habitats along the northeastern Atlantic seaboard (latitude 41°18'N to 44°56'N, longitude 70°16'W to 65°21'W) from 2000 to 2015. The majority of samples were opportunistically collected in the Bay of Fundy, Canada, where right whales congregate for feeding between July and October (Brown et al., 1995). Right whales of both sexes and all age groups were sampled for feces either by observing defecation at the surface (Rolland et al., 2005) or by using scent detection dogs trained to locate right whale feces (Rolland et al., 2006). A 300 µm nylon mesh dip-net (Sea-Gear, Melbourne, FL) attached to an extendable pole was used to scoop up floating fecal samples (Rolland et al., 2005). Fecal samples were transferred to a polypropylene jar and kept on icepacks in a cooler while in the field, before being stored temporarily at –20 °C and then archived at –80 °C. Date, time and location (latitude/longitude) of collection, and whale identification data were recorded.

Individual whales were identified by photographs of unique patterns of cornified epithelium (i.e., callosities) and permanent scars (Kraus et al., 1986) using the North Atlantic Right Whale Identification Database (North Atlantic Right Whale Consortium, 2016a). Alternatively, some fecal samples were associated with individual whales using genetic profiling of fecal DNA coupled with sighting history and hormone profiles (Gillett et al., 2010). Whales were categorized, based on whale age and reproductive history (Hamilton et al., 1998), as calves (<1 year old, associated with their mother, and likely nursing), juveniles (1–8 y.o. and never calved), adults (year before first calving or

187 ≥ 9 y.o.), resting females (parous, non-pregnant and not lactating), pregnant females

188 (confirmed by identification with newborn calf in the year following sampling), and

189 lactating females (with a dependent calf).

190

191 **2.2 Hormone extraction and analysis**

192 Fecal sample processing and hormone extraction methods followed Rolland et al. (2005).

193 Samples were lyophilized at –20 °C, sifted through a 1 mm stainless steel mesh, and

194 stirred until homogenous. To extract hormones, 0.2000 ± 0.01 g of the resulting powder

195 was mixed with 2.0 mL of 90% methanol in a borosilicate glass tube. Capped tubes were

196 vortexed for 30 min, centrifuged for 30 min at 2165 *g*, and 1.0 mL of the resulting

197 supernatant was aliquoted into a clean, airtight vial. Extracts were prepared no more than

198 two years prior to this study, and stored at –80 °C until hormone analysis.

199

200 Immunoreactive aldosterone was quantified in fecal extracts using a commercial solid-

201 phase ^{125}I radioimmunoassay kit for aldosterone (catalog #TKAL1 Coat-a-Count®,

202 Siemens Healthcare Diagnostics Inc., Norwood, MA, USA). This radioimmunoassay was

203 analytically validated for right whale fecal samples by testing for: (1) parallelism between

204 serial dilutions of pooled fecal extract (1:2–1:128) and the standard reference curve; and

205 (2) accuracy (i.e., matrix effect test) in results of standards spiked with pooled fecal

206 extract vs. unspiked standards (Diamandis and Christopoulos, 1996). In supplementary

207 trials to assess the suitability of commercially available kits for measuring aldosterone in

208 feces, two enzyme immunoassays were also tested for parallelism (catalog #11-ALDHU-

209 E01, Alpco, Salem, NH, USA and catalog #DEIA4474, Creative Diagnostics, Shirley,

210   NY, USA; see Results). For this study, we chose to proceed using the more sensitive

211   radioimmunoassay (analytical sensitivity of 11 pg/mL; cf. 15 pg/mL for enzyme

212   immunoassays), which has also been previously used to quantify serum aldosterone in

213   other cetacean studies (see Fair et al., 2014; Hart et al., 2015; Houser et al., 2011; St

214   Aubin et al., 2011). In brief, standards (25–1200 pg/mL), controls (high and low

215   concentrations) and fecal samples (extract diluted 1:16 in calibrator zero; part #ALC3)

216   were assayed in duplicate following the manufacturer's protocol, and counted for 1 min in

217   a gamma counter (Wallac 1470 WIZARD®; PerkinElmer Life Sciences, Waltham, MA,

218   USA). All samples, standards and controls were assayed in duplicate. Any sample with a

219   coefficient of variation between duplicates >10% was re-assayed.

220

221   Quality control in assays (i.e., precision and reproducibility) was monitored by measuring

222   aldosterone concentration of high (~30% binding) and low (~70% binding) control

223   samples in each assay (n = 13 assays). Inter-assay coefficients of variation were 2.7 ±

224   4.4% (high control) and 3.6 ± 0.3% (low control). Intra-assay coefficient of variation was

225   6.3 ± 1.2%. As reported by the manufacturer, the aldosterone antibody used had 100%

226   cross-reactivity with aldosterone and < 0.06% for spironolactone, 18-OH-corticosterone,

227   progesterone, 11-deoxycorticosterone, corticosterone, androsterone, DHEA, 11-

228   deoxycortisol, cortisone, dexamethasone and prednisolone (no detectable cross-reactivity

229   for cortisol, estradiol and testosterone). Final hormone data were expressed as nanograms

230   of immunoreactive hormone metabolites per gram of dry fecal powder.

231

232   **2.3 Statistical analyses**

233   Data on whale identity, sex and reproductive state (calf of both sexes; juvenile male;

234   mature male; juvenile female; resting female; pregnant female; and lactating female), as

235   well as season and region (Bay of Fundy and Roseway Basin in northern Gulf of Maine,

236   Canada; Great South Channel and Cape Cod Bay in southern Gulf of Maine, the United

237   States) were integrated with fALD results for all samples. Hormone concentrations were

238   transformed ($\log_{10}$) to adjust for a skewed, non-normal distribution. Levene tests were

239   conducted to check for homogeneity of variance. Descriptive statistics (mean ± SEM,

240   range) were used to summarize the data set. Concentration ranges reported for each

241   cohort were lower and upper 95th percentile intervals, which were calculated using a

242   normal distribution method (Altman, 1991) on transformed fALD data.

243

244   Concentrations of reproductive and stress hormones in fecal samples (i.e., metabolites of

245   testosterone, fT; progesterone, fP; estrogens (estradiol and estrone), fE; and

246   glucocorticoids, fGC) from the same whales sampled in this study have been presented

247   elsewhere (Hunt et al., 2006; Rolland et al., 2005), and were used here to interpret fALD

248   results. Data used to investigate possible effects of assay cross-reactivity in hormone

249   results involved all collected samples (i.e., samples from unknown individuals [n = 205

250   samples] and all samples from known individuals including repeated samples [n = 110

251   samples]). Outlier results for each hormone, i.e., values outside three times interquartile

252   range (Tukey, 1977), were excluded from analyses to prevent a disproportionate

253   influence of extreme values (39 samples were excluded). To analyze relationships

254   between fALD and other steroid hormone concentrations in whale feces, we used a

255   generalized linear model fitted by maximal likelihood with normal errors and an identity

256   link. A full factorial model was used to examine the effect of gonadal (i.e., fT, fP and fE)

257   and other adrenal (i.e., fGC) steroid hormones (predictor variables) on fALD

258   concentrations (response variable) for whale samples. Assumptions were tested by

259   visually checking residual distributions and Q-Q plots. Linear correlations were

260   performed to examine the relationship between fALD and other fecal steroid hormones.

261   We expected a strong correlation between fALD and fGCs because, although the assays

262   detect different hormones, these are both steroids produced by the adrenals and

263   influenced by ACTH secretion. However, correlations between fALD or fGC and fT, fP

264   or fE steroids might indicate that metabolites from other sources (i.e., gonads) are being

265   co-measured, affecting similarity of assay measurements (cross-reactivity).

266

267   Fecal aldosterone concentrations of individual right whales were compared across sex

268   and reproductive state using univariate ANOVA, with season and region included in the

269   analysis as these parameters can be influential factors (Goymann, 2012). For those

270   identified whales repeatedly sampled during the project, we used the data from the first

271   fecal sample collection only, to ensure that individual whales were represented only once

272   in these statistical analyses. However, to explore the variation in fALD within an

273   individual, we calculated the ratio of the difference between repeat sample concentrations

274   for those whales sampled on more than one occasion. Univariate ANOVA was used to

275   compare the change in fALD measures of individual right whales throughout various life

276   history stages (juvenile female to reproductively active adult; resting female to pregnant;

277   resting female to lactating; and whales with a consistent reproductive state between

278   sampling events). *Post hoc* Bonferroni tests of pairwise comparison were used to identify

279 all significant differences. All analyses were conducted using the statistical program

280 SPSS (version 22.0 for Macintosh, SPSS Inc., Chicago, IL, USA). A difference of $P <$

281 0.05 was considered significant for all statistical tests.

282

283 **3.0 RESULTS**

284 The radioimmunoassay used to quantify fALD was validated for North Atlantic right

285 whales by demonstrating: (1) close parallelism (linear regression, $F_{1,8} = 3.51$, $P = 0.10$),

286 indicating that hormone metabolites were being reliably measured across a range of

287 concentrations (Figure 1a); and (2) good accuracy ($R^2 = 0.99$, slope = 0.93 in accuracy

288 test plot), indicating that sample matrix does not interfere with antibody binding (Figure

289 1b). The other enzyme immunoassay assays tested in this study were also able to detect

290 quantifiable amounts of aldosterone metabolites and demonstrated reliable parallelism

291 with right whale fecal extracts (Alpco: $F_{1,9} = 0.05$, $P = 0.82$; and Creative Diagnostics:

292 $F_{1,8} = 0.82$, $P = 0.39$) but were not tested further.

293

294 Variation in fALD concentration among right whale samples was significantly associated

295 with the concentration of fGC (GLM fitted by maximal likelihood, $b = 0.33 \pm 0.06$, Wald

296 $\chi^2 = 34.76$, $P < 0.001$) and fP ($b = 0.13 \pm 0.05$, Wald $\chi^2 = 7.63$, $P = 0.01$), but not with fT

297 ($b = -0.03 \pm 0.03$, Wald $\chi^2 = 0.94$, $P = 0.33$) or fE ($b = 0.07 \pm 0.04$, Wald $\chi^2 = 3.84$, $P =$

298 0.05). Fecal aldosterone concentrations exhibited the strongest correlation with fGC ($r =$

299 0.59, $P < 0.001$), with levels of both these adrenal hormones increasing congruently

300 (Figure 2). A positive correlation was also found between fALD and gonadal hormones

301 (fT, fP and fE) in samples. However, in contrast to fGC, the gonadal hormones expressed

302 a weaker correlation with fALD (Table 1); whereas, fGC levels increased most strongly

303 in association with fT and fE concentrations in samples ($r = 0.67$ and $r = 0.66$

304 respectively, both $P < 0.001$; Table 1).

305

306 Across all samples collected (total n = 315), 82 individual North Atlantic right whales

307 (~15-25% of the estimated population; North Atlantic Right Whale Consortium, 2016b)

308 were represented (32 males and 50 females). The dataset of known whales included three

309 calves (two males and one female), 10 juvenile males, 12 juvenile females, 20 adult

310 males, 15 resting females, eight pregnant females, and 14 lactating females. Most fecal

311 samples were collected from right whales in the northern habitats of Bay of Fundy and

312 Roseway Basin over summer (n = 39 individuals) and fall (n = 38), with a few samples

313 from the Great South Channel and Cape Cod Bay in spring (n = 5). No samples were

314 collected in winter when most right whale locations are unknown, and those whales in the

315 calving ground appear to be fasting.

316

317 Fecal aldosterone concentrations in right whales ranged from non-detectable (n = 4) to

318 75.8 ng/g, with the vast majority of samples (90%) ranging between 2.7–21.5 ng/g (i.e.,

319 lower and upper 95th percentile interval). Overall, fALD levels were similar in males (8.0

320 ± 0.7 ng/g) and females (11.7 ± 2.0 ng/g) (ANOVA, $F_{1,69} = 0.26$, $P = 0.61$). However,

321 concentrations of fALD were significantly associated with reproductive state ($F_{6,69} =$

322 9.39, $P < 0.001$). Pregnant females had the highest fALD (35.9 ± 7.6 ng/g, 9.6–98.3 ng/g;

323 $P < 0.05$), followed by reproductively mature males (9.5 ± 0.9 ng/g, 2.9–24.6 ng/g; $P <$

324 0.05), compared to all other whales (Figure 3). The lowest fALD concentrations were in

325   immature individuals of both sexes (males, 5.1 ± 1.2 ng/g and females, 5.6 ± 1.0 ng/g;

326   both sexes, 1.0–19.0 ng/g), unweaned yearling calves (6.7 ± 1.2 ng/g, 2.3–12.3 ng/g), as

327   well as lactating (7.4 ± 0.7 ng/g, 3.7–13.6 ng/g) and resting females (8.1 ± 1.2 ng/g,

328   2.9–18.4 ng/g) (all $P > 0.05$). Sampling location ($F_{3,69} = 0.73$, $P = 0.54$) and season ($F_{2,69}$

329     $= 0.05$, $P = 0.96$) did not significantly influence fALD concentrations in this study, with

330   similar levels found among whales in the northern habitats over summer (11.8 ± 2.3 ng/g)

331   and fall (9.3 ± 1.2 ng/g) and southern habitats over spring (5.8 ± 1.6 ng/g).

332

333   The fALD results across the population were comparable to concentration changes within

334   individual whales that were repeatedly sampled across different reproductive states. The

335   only significant change in fALD concentration among the individuals examined was an

336   increase exhibited by females with pregnancy (ANOVA, $F_{3,21} = 8.53$, $P = 0.001$). These

337   adult females had low fALD levels during a resting phase (7.4, 5.1, and 4.4 ng/g) that

338   increased four-fold and up to 16-fold during pregnancy (35.5, 24.5, and 75.8, ng/g,

339   respectively). Lactating females (n = 3) had negligible changes in fALD concentration

340   compared to their resting phase (from 8.3 at resting to 7.7 ng/g during lactation; 5.9 to 8.2

341   ng/g; and 4.5 to 9.0 ng/g; $P > 0.05$). Two females sampled both as juveniles and then as

342   adults, initially had low fALD concentration that increased by one- and three-fold once

343   reproductively mature (from 6.0 to 10.0 ng/g; and 5.7 to 23.2; $P > 0.05$). Individual

344   whales that were repeatedly sampled in a consistent life history stage, as a juvenile (n =

345   4), adult male (n = 4), resting female (n = 2) or during lactation (n = 4), had similar fALD

346   concentrations between temporal samples while in the same reproductive state, i.e., less

347   than 1-fold change in concentration ($P > 0.05$).

348

4.0 DISCUSSION

Fecal aldosterone concentrations reported here provide the first data on this hormone class for a free-swimming whale in its natural environment. For large whales, aldosterone has previously only been measured in postmortem serum samples from hunted fin whales (*Balaenoptera physalus*) (Kjeld, 2001). Our study demonstrated that aldosterone, a mineralocorticoid functioning in the mammalian stress response (Kubzansky and Adler, 2010; Romero and Wingfield, 2016), can be quantified in feces of North Atlantic right whales using commercially available immunoassays. Furthermore, all three of the antibodies tested in this study detected immunoreactive aldosterone metabolites in right whale feces. Archived fecal samples collected from right whales over the last decade presented a unique resource for physiologic study that is difficult to obtain from a large whale species. Similar to other steroids, aldosterone levels in feces are likely to represent an accumulation of metabolized hormone over the duration of gastrointestinal transit, which is estimated to be 1-2 days for most large-bodied mammals (reviewed by Schwarzenberger et al., 1996) including right whales (Rolland et al., 2005). The presence of mineralocorticoids in feces has been investigated in laboratory rats (Morris et al., 1976) but not among wildlife species (for which aldosterone has only been quantified with blood measures), and could provide an informative biomarker for other endocrine studies using fecal samples.

368

Measuring fALD allowed for a more comprehensive interpretation of adrenal activation for certain whales, especially in relation to reproductive condition. Previously, only fGC

has been used to assess adrenal function in right whales, but data can be confounded by potential measurement of other cross-reacting steroids (Hunt et al., 2006). The inability to conduct radiometabolism studies in large whales makes it difficult to discern whether fGC results for particular fecal samples are reflective of adrenal activation influenced by active reproductive condition or an artefact of cross-reactivity with gonadal hormone metabolites. The antibody used to quantify aldosterone had extremely low cross-reactivity with other steroids (as tested by the manufacturer); and likewise, the fGC antibody had only 0.03% cross-reactivity with aldosterone (Hunt et al., 2006). Results showed that fALD concentrations were more strongly associated with fGC, as a complementary adrenal hormone, than to other gonadal steroid concentrations. The correlation among adrenal hormones suggests that ACTH induced the stimulation of these adrenocorticoids (Schmitt et al., 2010; Ortiz and Worthy, 2001; Thomson and Geraci, 1986), and that some whales were exhibiting a stress response with elevations of both fGC and fALD.

Free-swimming right whales had fALD concentrations within a relatively low and narrow (seven-fold) concentration range (2.7–21.5 ng/g) compared to other fecal hormone metabolites reported for this species, such as androgens (~1000–16,000 ng/g), progestagens (~100–200,000 ng/g), estrogens (~35–40,000 ng/g) and glucocorticoids (~10–10,000 ng/g) (Hunt et al., 2006; Rolland et al., 2005, in prep). Studies measuring serum or plasma aldosterone of small cetaceans also reported comparatively low concentrations, with values ranging between ~0.005 and 0.04 ng/mL in bottlenose dolphins (Houser et al., 2011) and belugas (Schmitt et al., 2010) held in captivity and

394   behaviorally conditioned for blood sampling; and up to 0.5 ng/mL in captured and

395   restrained wild bottlenose dolphins (Hart et al., 2015; Fair et al., 2014; Ortiz and Worthy,

396   2000; St. Aubin et al., 1996), pantropical spotted dolphins (*Stenella attenuata*) (St Aubin

397   et al., 2013) and belugas (St. Aubin and Geraci, 1989). Although aldosterone is likely

398   maintained within a restricted physiological range in circulation, our study found intrinsic

399   differences in fALD with reproductive maturity and condition of whales. These overall

400   patterns of fALD release were consistent with those reported for fGC in right whales

401   (Hunt et al., 2006), such that relatively higher concentrations of both adrenal hormones

402   were found in pregnant females, as well as reproductively mature males. This

403   correspondence between GC and mineralocorticoid activity across reproductive cohorts

404   indicates concurrent secretion of these hormones by the adrenal gland in this species.

405   These patterns likely reflect normal physiologic responses to changing energetic

406   expenditure and metabolic needs with reproductive condition. Whales with the lowest

407   fALD concentrations were sexually immature individuals and all adult females in a non-

408   pregnant phase. Nursing calves had equivalent fALD concentrations to lactating females;

409   although cows and calves measured in this study were not related, the similarity in fALD

410   levels between these groups may suggest some hormonal transfer via milk fat during

411   nursing (Safwate et al., 1981).

412

413   The addition of fALD aids interpretation of adrenal activation by providing supportive

414   evidence that adult males had nearly twice the concentration of this stress-related

415   hormone compared to immature males. Given the shared chemical structure of most

416   androgen and GC metabolites (e.g., androstrane; Ganswindt et al., 2003) and the

417   magnitude of fT levels in reproductively mature males as well as pregnant females

418   (Rolland et al., 2005), the potential measurement of cross-reacting gonadal testosterone

419   metabolites represents a concern when applying fGC assays in particular samples (Hunt

420   et al., 2006). The need for care when interpreting fGC in individuals with highly skewed

421   androgen levels was highlighted for right whales by Hunt et al. (2006), and demonstrated

422   for male African elephants (Ganswindt et al., 2003) and male dogs (Schalz and Palme,

423   2001). However, the combined dataset of fGC and fALD shows that male right whales

424   appear to be genuinely expressing an elevated stress response coincident with

425   reproductive maturity. Mature males can experience increased reproductive competition,

426   requiring mobilization of fuel sources and associated stress responses (Boonstra, 2005),

427   which is plausible for male right whales given the rigorous surface-active courtship

428   behavior of this species (Kraus and Hatch, 2001), sometimes observed when surveying

429   right whales in the northern Gulf of Maine (Rolland et al., 2005). An increase of

430   aldosterone production and function of the renin-angiotensin system could enable

431   reproductively active males to conserve sodium, increase water retention and blood

432   pressure, and maintain blood flow during heightened physical activity (Lieu et al., 2014).

433

434   Pregnant females had the highest fALD concentrations among all demographic groups,

435   which was also reported for fGC in right whales (Hunt et al., 2006). With pregnancy,

436   parous females typically had an increase in fALD concentration that was four-fold higher

437   than resting condition levels; although one individual female (sampled twice over two

438   years) showed a substantial 16-fold increase in her fALD levels accompanying

439   pregnancy. Pregnancy involves a series of metabolic and cardiovascular adjustments, the

440   aim of which is to maintain a constant energy supply for fetal development (Challis et al.,

441   2011). For pregnant whales, apparently normal endocrine responses reported so far

442   include highly elevated production of progesterone to help establish and maintain

443   pregnancy (Rolland et al., 2005), but also elevated GCs (Hunt et al., 2006) and

444   mineralocorticoids (present study). Increased concentrations of adrenal hormone

445   metabolites (both fGC and fALD) in the feces of pregnant whales is not unexpected,

446   because the maternal adrenal gland is active in response to endocrine changes throughout

447   gestation (Challis et al., 2011; Robeck et al., 2017); and such biologically relevant

448   differences in hormone production with pregnancy can be detected in feces (e.g., fGC;

449   Burgess et al., 2013; Foley et al., 2001). Aldosterone levels in humans are known to

450   increase as pregnancy progresses to help maintain sodium balance and blood pressure,

451   especially considering the natriuretic effects of progesterone and other gestational factors

452   that cause an excessive loss of sodium (Elsheikh et al., 2001). Like other mammals,

453   activation of the renin-angiostensin system and circulating aldosterone is likely to play

454   vitally important roles during gestation in right whales, by serving the increased demand

455   for salt, and hence water, and facilitating the expanding needs of blood supply and

456   nutrients for both mother and fetus (Irani and Xia, 2008).

457

458   Fecal aldosterone levels have not been reported for wildlife species, even terrestrial taxa.

459   To enhance the use of fALD assays, we recommend that studies aim to identify the

460   immunoreactive metabolites of aldosterone present in feces. Even in the absence of such

461   validations, simultaneous analyses of two alternative stress-related adrenal hormones

462   (i.e., GCs and mineralocorticoids), and potentially other metabolic hormones such as

463   thyroid hormones (e.g., triiodothyronine, also known to be present in mammalian feces;

464   Wasser et al., 2010), may aid identification of physiologic responses when using

465   noninvasive fecal samples. Mineralcorticoid data could complement the extensive

466   application of fGC in stress assessment studies, and help evaluate confounding factors

467   especially relevant for species for which radiolabelled infusion validations are logistically

468   impossible. Moreover, each of these hormones may exhibit differing physiologic

469   responses to various intrinsic or extrinsic stressors, such that comprehensive study of all

470   stress-associated hormones might enable discrimination of the relative impact of multiple

471   co-occurring pressures (e.g., vessel disturbance vs. food availability; Ayres et al., 2012).

472   Future studies of aldosterone in whales in compromised health, poor body condition, or

473   exposed to a known stressor (such as anthropogenic noise) will allow us to better

474   understand changes in aldosterone secretion, and its conservation relevance. In addition,

475   fALD could also be useful for studies focusing on osmotic regulation, which is so vital to

476   marine mammal physiology (Ortiz, 2001) - and might yield critical insights into marine

477   mammal responses in the face of ocean salinity changes with global warming (Curry et

478   al., 2003). Given the complexity of anthropogenic disturbances and environmental

479   challenges faced by marine wildlife (Davidson et al., 2012; Schipper et al., 2008), the

480   addition of fALD as a biomarker of adrenal activation may help partition the causes of

481   stress hormone elevations in large whales.

482

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691 Figure 1. Validation plots for measuring aldosterone in fecal extracts from North Atlantic
692 right whales using radioimmunoassay. Note: close parallelism (a) between serially diluted
693 samples (dilutions 1:2 through 1:64; open circles) to the aldosterone standard curve (25–
694 1200 pg/mL; closed circles) ($P = 0.10$); and good accuracy (b) demonstrated by the
695 positive linear relationship of known aldosterone concentration against apparent
696 concentration in spiked samples ($R^2 = 0.99$), with a slope of approximately 1.0 (dotted
697 line; $y = 31.39 + 0.93x$).
698
699
700 Figure 2. Concentrations (ng/g, plotted on a logarithmic scale) of immunoreactive
701 aldosterone (fALD) and glucocorticoids (fGC) in feces of North Atlantic right whales,
702 correlated within samples (n = 276). Dashed trend line represents least-squares linear
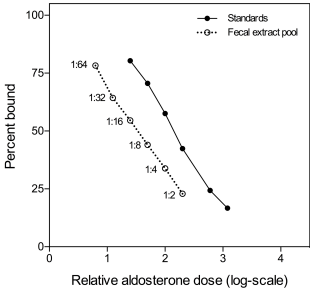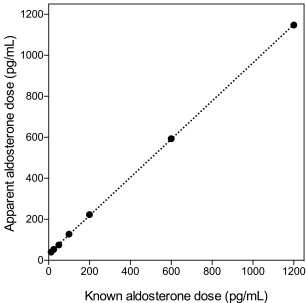703 regression fit to the data set, $y = 0.16 + 0.45x$ ($P < 0.001$).
704
705
706 Table 1. Correlation matrix for fecal aldosterone (fALD) and fecal glucocorticoid (fGC)
707 concentrations with reproductive hormones (i.e., fecal testosterone, fT; fecal
708 progesterone, fP; and fecal estrogens, fE). Values represent calculated Pearson product-
709 moment correlation coefficients (r), as a measure of linear dependence between two
710 variables (where $r = 1$ is total positive correlation). Highlighted in bold are stronger
711 associations between fALD, fGC and reproductive hormones (i.e., $r \geq 0.5$). Double
712 asterisks indicate a significant relationship between hormone concentrations at $P < 0.001$.
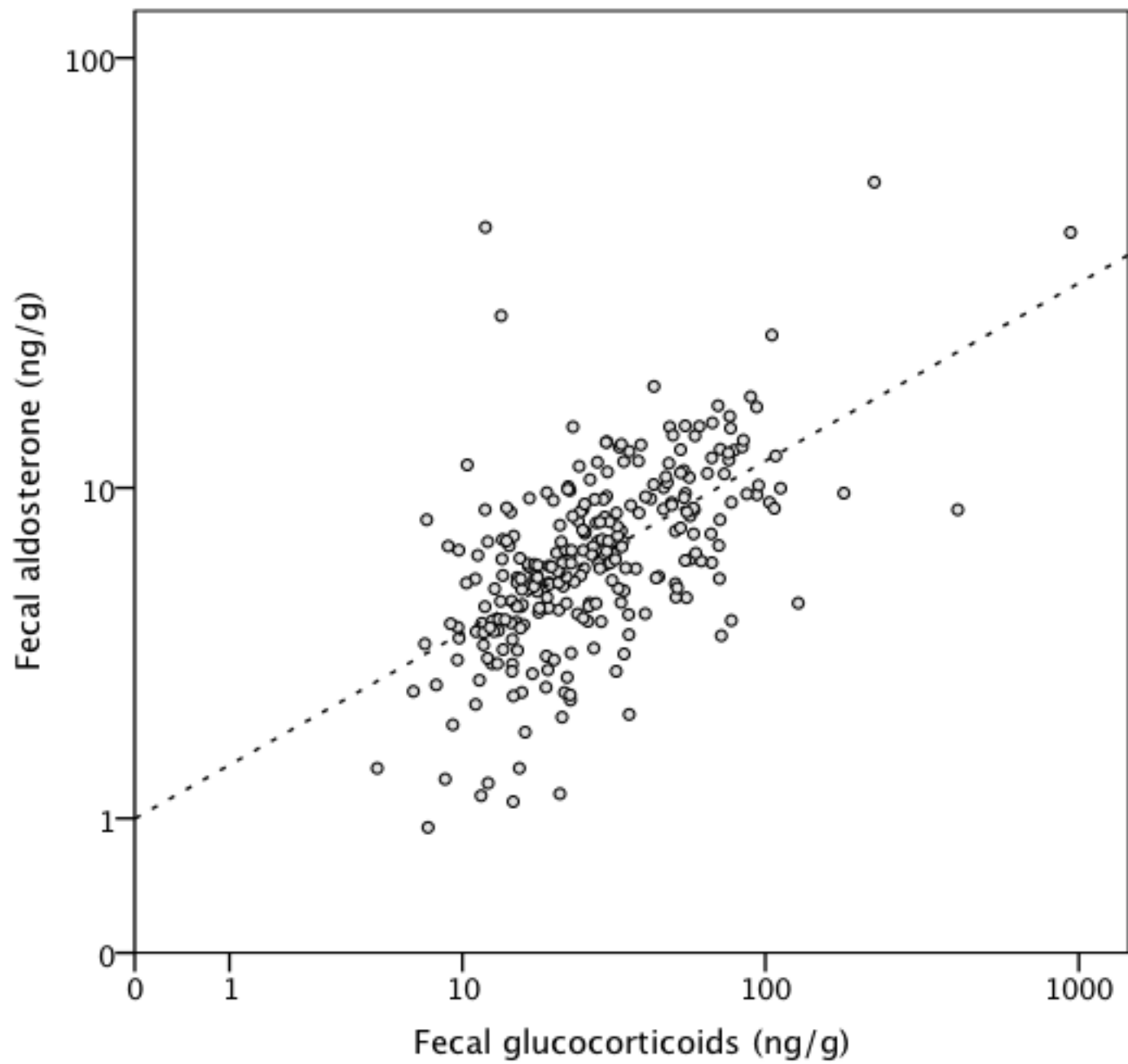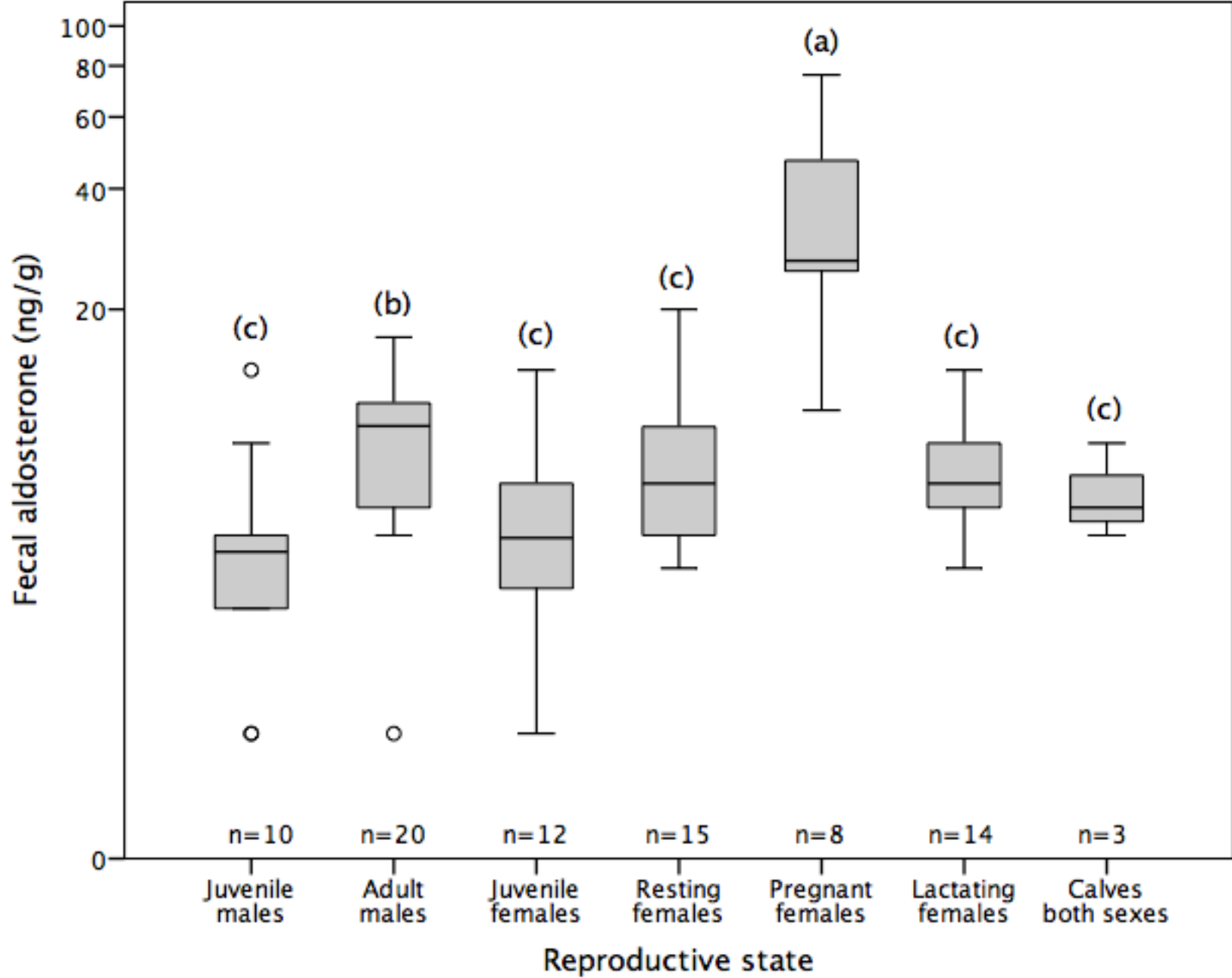713
714
715 Figure 3. Differences in fecal mineralocorticoid concentrations (ng/g, plotted on a
716 logarithmic scale) in photo-identified North Atlantic right whales (total n = 82
717 individuals), according to sex and reproductive state. For boxplots, the line inside the box
718 indicates the median value, the height of the box encompasses the distance between the
719 25th and 75th quartiles, and the whiskers delineate extreme observations. Outliers are
720 marked with an open circle (>1.5 × interquartile range). Different letters denote a
721 significant difference in resulting hormone measures between reproductive groups at $P <$
722 0.05.

Legend:
— Standards
○ Fecal extract pool

X-axis: Relative aldosterone dose (log-scale)
Y-axis: Percent bound

Dilution labels: 1:64, 1:32, 1:16, 1:8, 1:4, 1:2

	fALD	fGC
fGC	**0.59 ****	**0.59 ****
fT	0.38 **	**0.67 ****
fP	0.42 **	**0.50 ****
fE	0.42 **	**0.66 ****