JOURNAL OF EXPERIMENTAL ZOOLOGY 299A:188–196 (2003)

Fecal Corticosterone Assessment in the Epaulette Shark, *Hemiscyllium ocellatum*

AMANDA H. KARSTEN* AND JOHN W. TURNER, JR.*
Medical College of Ohio, Department of Physiology and Molecular Medicine, Toledo, Ohio 43614–5804

ABSTRACT The present study examined the feasibility of measuring the steroid hormone corticosterone in fecal extracts of epaulette sharks, *Hemiscyllium ocellatum*. Six immature, captive-raised epaulette sharks (four females and two males) were obtained from two different zoos and were maintained in a closed-system, 530–liter aquarium. After a one-month adaptation, fecal samples were collected daily from each animal for 33 days. Five-day sets of samples were pooled within animals to insure sufficient material for analysis. Fecal hormone extraction was achieved using repeated cycles of dichloromethane and aqueous washes. The levels of corticosterone were measured by reverse-phase high-performance liquid chromatography (HPLC). Corticosterone presence in HPLC eluent peaks from fecal extracts was determined by comparison of the elution pattern of corticosterone standard with the elution patterns of fecal extracts with and without the addition of tritiated corticosterone or exogenous, unlabeled corticosterone. Exclusive presence of corticosterone in HPLC eluent peaks presumed to be corticosterone was determined by nuclear magnetic resonance mass spectrometry. Corticosterone levels, calculated from a 10–point standard curve, ranged from 1.2 to 20.9 ng/g feces across all sharks, with 92.3% of values being $\leq$13.5 ng/g. Within individuals, the lowest average for corticosterone levels across 33 days was 2.6 ± 0.4 ng/g feces, and the highest average was 8.4 ± 2.2 ng/g feces. This study demonstrated that corticosterone was extractable from and reliably measurable in fecal extracts of epaulette sharks. This is the first evidence of this hormone in epaulette sharks and the first report of fecal corticosterone in elasmobranchs. *J. Exp. Zool. 299A:188–196, 2003.*

INTRODUCTION

In the face of ecological compromises associated with marine environments in recent years (Rogers et al., '94; Carpenter et al., '98; Abelson et al., '99) assessment of stress in marine vertebrates may be useful. Glucocorticoids have been employed as indicators of stress (Asterita, '85) and have been measured in fish (Barton and Iwama, '91; Bonga and Wendelaar, '97; Grutter and Pankhurst, 2000) and elasmobranchs (Rasmussen and Crow, '93; Rasmussen and Gruber, '93; Snelson et al., '97). Studies to date have utilized plasma or serum for glucocorticoid assessment. However, invasive techniques of blood collection are in themselves stressful and have been found to increase the levels of corticosteroids (Bassett and Hinks, '69; Stephens, '80).

An alternative means for obtaining information regarding glucocorticoids is via analysis of fecal extracts. Miller et al. ('91) used this noninvasive approach to measure stress in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*). Fecal glucocorticoids have also been measured in a variety of other mammals (Whitten, '97; Jurke et al., '97; Goymann et al., '99; Wasser et al., 2000; Turner et al., 2002) and in birds (Wasser and Starling, '88). Glucocorticoids are only sparingly soluble in water, therefore, their measurement in fecal casts of marine animals may be feasible, if these casts can be identified and collected.

The present study was undertaken to determine the feasibility and reliability of measuring the stress-related hormone, corticosterone, in extracts of feces obtained in captivity from the reef-

Dr. Karsten's current address is: Clemson University, Pendelton, South Carolina, 29670–0709
Grant sponsor: MCO Research Incentive Program; Grant number 96850004
*Correspondence to: Amanda H. Karsten, Clemson University, Department of Environmental Toxicology, 509 Westinghouse Road, P.O. Box 709, Pendleton, SC 29670–0709. E-mail: hkamand@clemson.edu; or: John W. Turner, Medical College of Ohio, Department of Physiology and Molecular Medicine, Toledo, Ohio, 43614–5804. E-mail: jturner@mco.edu
Received 11 February 2003; Accepted 27 June 2003
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/jez.a.10300

dependent epaulette shark (*Hemiscyllium ocellatum*).

MATERIALS AND METHODS

This study was performed under auspices of animal-use protocol (IACUC #100679) at the Medical College of Ohio. The epaulette shark is a carnivorous species found off the coast of Australia and New Guinea (Dingerkus and DeFino, '83; Dingerkus, '91; Last and Stevens, '94; Heupel and Bennett, '98). Advantages of this shark species for the present study include its ready adaptation to captivity (i.e., opportunity to observe basal corticosterone levels) and small size (<50 cm in this study), compared to other shark species. Six epaulette sharks (four females and two males) were used. Four of these animals were born in January, 1998, at the Florida Aquarium and two were born at the Columbus Zoo in January, 1998. All subjects were sexually immature, a circumstance which minimized the possibility of confounding effects of sex steroids in corticosterone assessment (Heupel et al., '99).

The animals were placed in a 530–liter, closed-system, salt-water aquarium in conditions similar to their previous aquarium environments. Water was tested every one to three days to monitor salinity (30–32 ppt), ammonia (<0.1 mg/l), temperature (65°–70°F) and pH (8.1–8.4). The animals experienced a 12–hr light/dark cycle and were fed a mixed diet of shrimp, scallops, krill, and smelt five days per week. Food consumption approximated 1% of body weight. The aquarium was divided into six equal sections by plastic mesh, separating individuals and permitting separate collection of feces from each shark. Fecal casts were solid, well-formed spirals and were collected from each animal daily for 33 days and were frozen at −40°C until extracted and assayed. Up to five days of fecal samples from each animal were combined to ensure that there was sufficient hormone for analysis.

All samples were extracted as described below. Four ml of fecal slurry (2 ml of tank water and 2 ml of fecal material) was homogenized and added to a plastic scintillation vial containing 12 ml of dichloromethane ($MeCl_2$). The mixture was mixed for 60 min, followed by 10 min centrifugation at 2000 g. The $MeCl_2$ layer was removed and shaken with 1 ml of 0.10 N NaOH for 2 min. The $MeCl_2$ layer was then removed and mixed with 1 ml of 18 megaohm water for 2 min. This layer was removed, passed through a 0.45 μm syringe filter, vacuum dried and stored in a −40°C freezer.

Samples were thawed and reconstituted in 50 μl of 100% acetonitrile (ACN) for 10 min. Water (18 megaohm) was added in three portions totaling 450 μl (with 2 min. of mixing per portion). One-half of the final 500 μl reconstituted sample was injected into a reverse-phase high-performance liquid chromatography (RP-HPLC model 300, Dionex Corporation, Sunnyvale, CA) attached to a C–18 column (Waters Corp., Milford, MA) and a variable-wavelength UV detector locked at a wavelength of 240 μ (Dionex Corporation, Sunnyvale, CA). The procedure utilized a water blank to establish stable baseline and a corticosterone standard at the beginning, middle, and end of each fecal-extract set.

Sample hormone concentration was based on UV absorbance of the area under the elution peak for corticosterone. Each corticosterone concentration was calculated from a 10–point standard curve (range 1.0 to 100.0 ng). The correlation coefficient for actual values vs. calculated logarithmic curve was 0.992. Hormone values are reported as ng/g feces (wet weight), and the lower limit of corticosterone detection was 1.0 ng per sample. Variation in the HPLC analysis was determined by measuring elution-peak times and elution-peak concentrations of corticosterone in seven aliquots of the same pooled shark fecal extract.

Some fecal samples were in the water up to 12 hours before collection. Therefore it was necessary to determine whether biodegradation of corticosterone occurred. Four grams of pooled feces was slurried in 4 ml of aquarium water, and the slurry was spiked with tritiated corticosterone (2.1×10^5 counts per min). One-half of the slurry was immediately extracted, and the other half was incubated at 27°C for 12 hrs and then extracted. Both aliquots were run on HPLC and the eluent peaks presumptive for corticosterone were mixed with Sigma Fluor cocktail (Sigma, St. Louis, MO) for aqueous samples. Radioactivity was determined in a Beckman LS 6500 liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA).

To support the identity of corticosterone in HPLC eluents, tritiated corticosterone (ICN Pharmaceuticals, Inc. Costa Mesa, CA) was used in initial HPLC elution experiments. A 0.1 ml aliquot of the tritiated corticosterone was added to scintillation fluid and measured for radioactivity as above. Aliquots of this tritiated corticosterone were then added to non-radioactive corticosterone

TABLE 1 Comparison of HPLC elution pattern of corticosterone standard and fecal extract using tritiated corticosterone as a spike

ELUTION TIME[a] (min)	PERCENT OF TOTAL TRITIUM SPIKE[b]	
	STANDARD	FECAL EXTRACT
16	1.8	2.0
17	0.8	3.1
18	2.3	3.1
19	88.1	87.3
20	5.3	2.9
21	1.0	0.8
22	0.7	0.6

[a]Each eluent consisted of a 1 min collection. Only collection periods containing radioactivity are shown.

[b]Tritium-spiked standard contained 919886 CPM total and tritium-spiked fecal extact contained 28805 CPM total as determined by liquid scintillation.

and to reconstituted fecal extract. These samples were analyzed by HPLC, and presumptive corticosterone-containing eluents were counted as above (Table 1).

To confirm that the presumptive corticosterone elution peak was in fact corticosterone and not 1α-hydroxycorticosterone, a closely related hormone in sharks, nuclear magnetic resonance (NMR) spectrometry was performed (Y. Kim, Univ. Toledo, Instrumentation Center). Our corticosterone standard (5 μg/ml, Sigma, St. Louis, MO) was analyzed as a reference compound. Pooling and concentration of elution peaks at 18.5 — 20.5 min from 30 fecal extracts produced sufficient material for complete analysis by NMR spectrometry (Covey, '96) as described in Results.

In cases where variation is reported, values are presented as mean ± standard error of the mean (SEM). For hormone measurement in individual sharks, each reported hormone value was determined from a pooled sample for each shark across five days of collection. The means and standard error of the mean (SEM) for these five-day pools were determined for each individual over the 33–day sampling period. Due to the small sample size, both within and between individuals, statistical analysis was not employed.

RESULTS

Corticosterone standard showed a consistently sharp HPLC elution peak between 19.55 and 19.66 (ave. 19.61). Several fecal samples were spiked with the corticosterone standard and showed an elution peak similar to the standard i.e., between 19.41 and 19.60 min with an average of 19.52 min. Representative elution profiles for an unlabeled, spiked fecal extract and an unspiked fecal extract are presented in Figure 1.

The fecal sample spiked with tritiated corticosterone and the tritiated corticosterone standard alone showed HPLC retention times of 19.58 and 19.60 min, respectively, suggesting reliable measurement of corticosterone by HPLC and noninterference by fecal constituents in corticosterone measurement (Table 1). The nearest adjacent elution peaks to corticosterone in sample extracts were >0.6 min away and unlikely to interfere with or be mistaken for corticosterone.

Variation among seven repeated aliquots of the same sample was 19.55 ± 0.26 min for the corticosterone elution peak and 4.8 ± 0.2 ng/g feces for the corticosterone concentration. Recovery of either unlabeled or tritiated corticosterone added to fecal samples ranged from 90–92%.

NMR spectrometry analysis performed on HPLC eluents for corticosterone standard, fecal extract eluting at 19.6 min and a pool of eluents from 30 fecal extract revealed that: 1) the molecular weight of standard corticosterone was 347.3 (Figure 2A), 2) the molecular weight of the fecal-extract peak eluting at 19.6 min was 347.2 (Figure 2B), and 3) fecal-extract eluents (i.e., the pool of eluents from 18.5–20.5 min) contained corticosterone, i.e., molecular wt. 347.1 was present (Figure 3). 1α-hydroxycorticosterone, which has a molecular weight of 363.1, appeared to be absent, although the mass spectrometry of the extract eluent between 18.5 and 20.5 min did contain a peak at 363.2 (Figure 3).

Further resolution of the structure of the ''unknown'' compound was obtained by chemical rendering (''fractionation'') of the compound into its respective atoms. The fractionation pattern of a corticosterone standard (5 μg) formed three distinct peaks (329.2, 311.2, 293.2) at an intensity range (based on the number of ions detected) of 6.0×10^4 to 21.0×10^4 intens (Figure 2A). Each peak represents a different fragment of the corticosterone molecule (J. Gano, pers. commun.). The fractionation pattern of the fecal-extract eluted from the HPLC at 19.6 min showed the same 329.2 peak found in the standard, which was consistent with corticosterone but not with 1α-hydroxycorticosterone. The 311.2 and 293.2 peaks seen in the standard were not detected in the extract. This was due to the low level of corticosterone in the extract relative to the standard. The intensity of the 329.2 peak in the

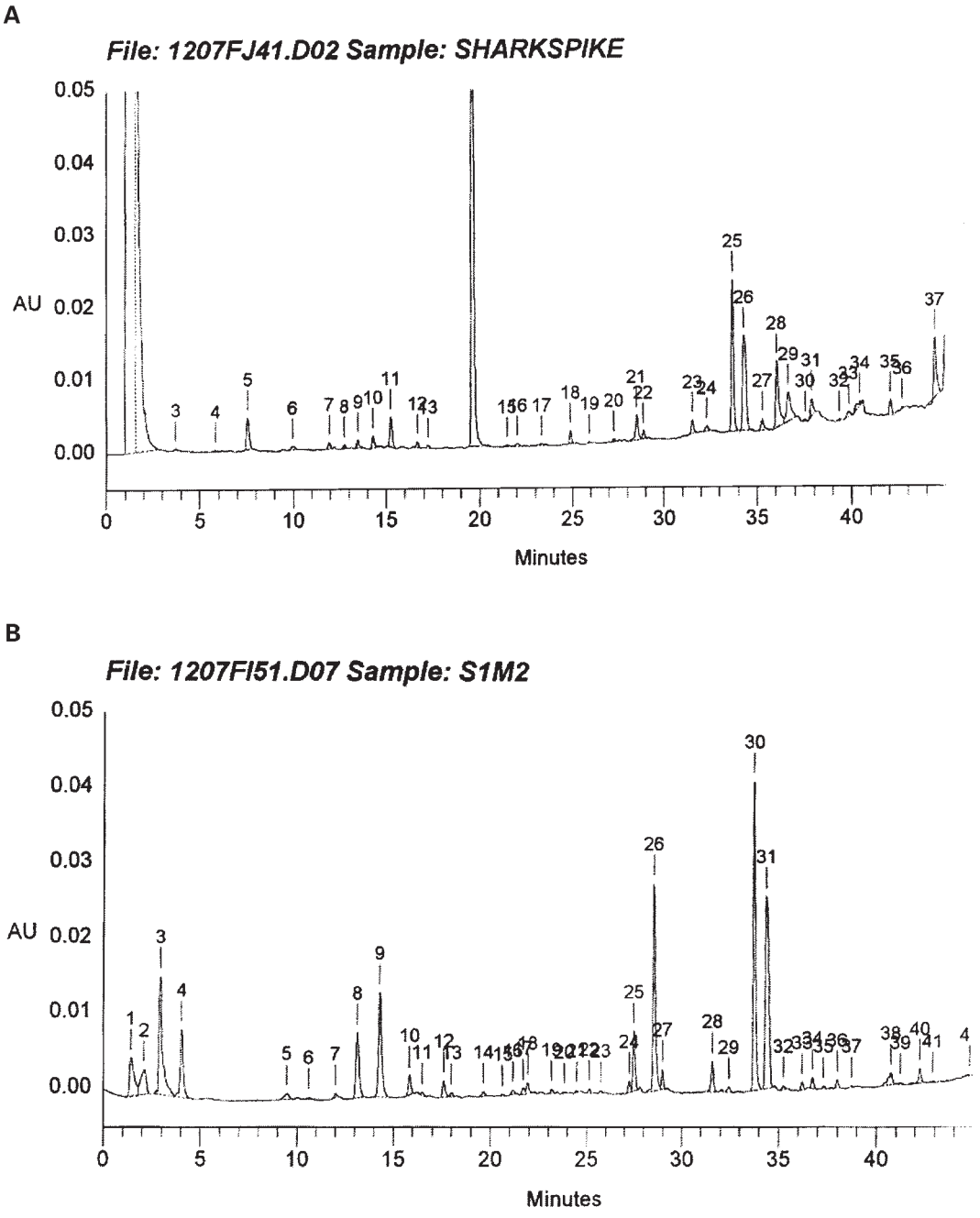

Fig. 1 HPLC elution profiles of two representative epaulette shark fecal extracts. Corticosterone eluted at 19.6 min, which is peak #14 in each sample. Sample A extract was spiked with 1 μg of corticosterone standard, and Sample B extract was unspiked.

extract was $<0.001\%$ of that for the 329.2 peak in the standard.

In the biodegradation test of fecal samples employing a tritiated-corticosterone spike of a pooled fecal sample, radioactivity in the HPLC eluent peak for corticosterone was 5877 CPM and 6401 CPM at zero and 12 hrs of incubation, respectively. These values differed by $<10\%$.

Among all samples collected from all six sharks during the 33–day sampling period, corticosterone ranged from 1.2 to 20.9 ng/g feces (Fig. 4). Values above 13.5 ng/g feces were observed in only 7.7% of samples. The greatest range of within-individual hormone concentration (5–day averages) across the 33–day collection period was from 2.0–20.9 ng/g feces and the smallest range was 1.7–4.5 ng/g

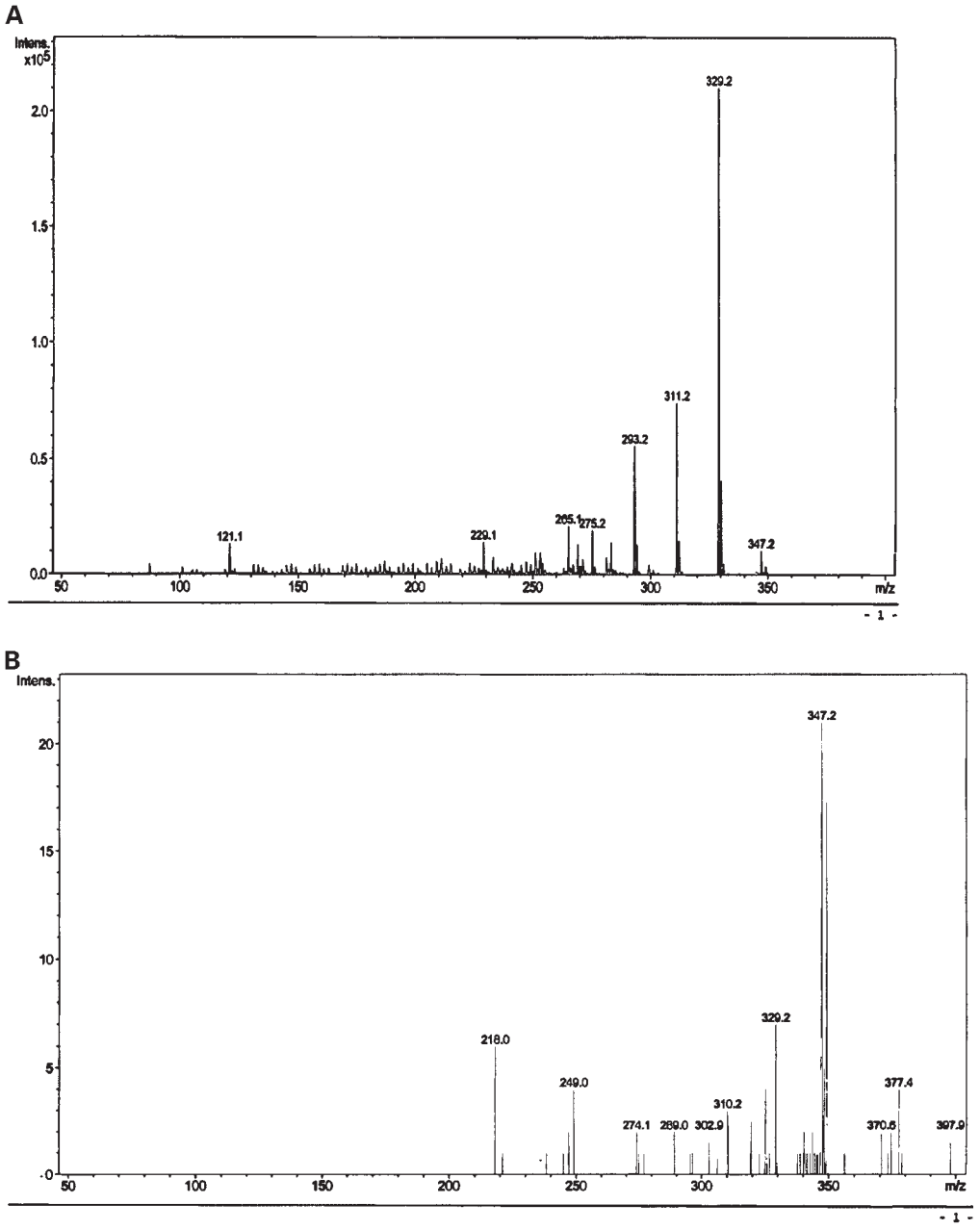

Fig. 2. Mass spectrometry fractionation pattern of: A) a 5 μg corticosterone standard showing molecular weight of 347.2 and component peaks at 293.2, 311.2 and 329.2, consistent with corticosterone structure, B) HPLC eluent of fecal-extract, using the presumptive corticosterone eluent peak (19.6) as the source. The predominant compound is 347. 1 molecular weight (corticosterone), and the fragment at 329.2 s consistent with corticosterone structure.

feces. The mean hormone concentration (ng/g feces) within a given individual (i.e., across time) was: female A=7.9±2.6, female B=2.6±0.5, female C=8.4±2.2, female D=3.9±1.1, male A=7.8±2.7 and male B=2.6±0.4. Water quality conditions were monitored biweekly to minimize possible physical/chemical stressors in captivity. Conditions monitored were salinity (30–32 ppt), ammonia (<0.1 mg/l), temperature (65°–70°F) and pH (8.1–8.4). Daily observations of individual animals were made for visible signs of stress, including rapid respiration, color changes, lack of appetite, and unresponsiveness. These signs, which were apparent when the animals arrived at the laboratory, were not present after acclimation and during the study.

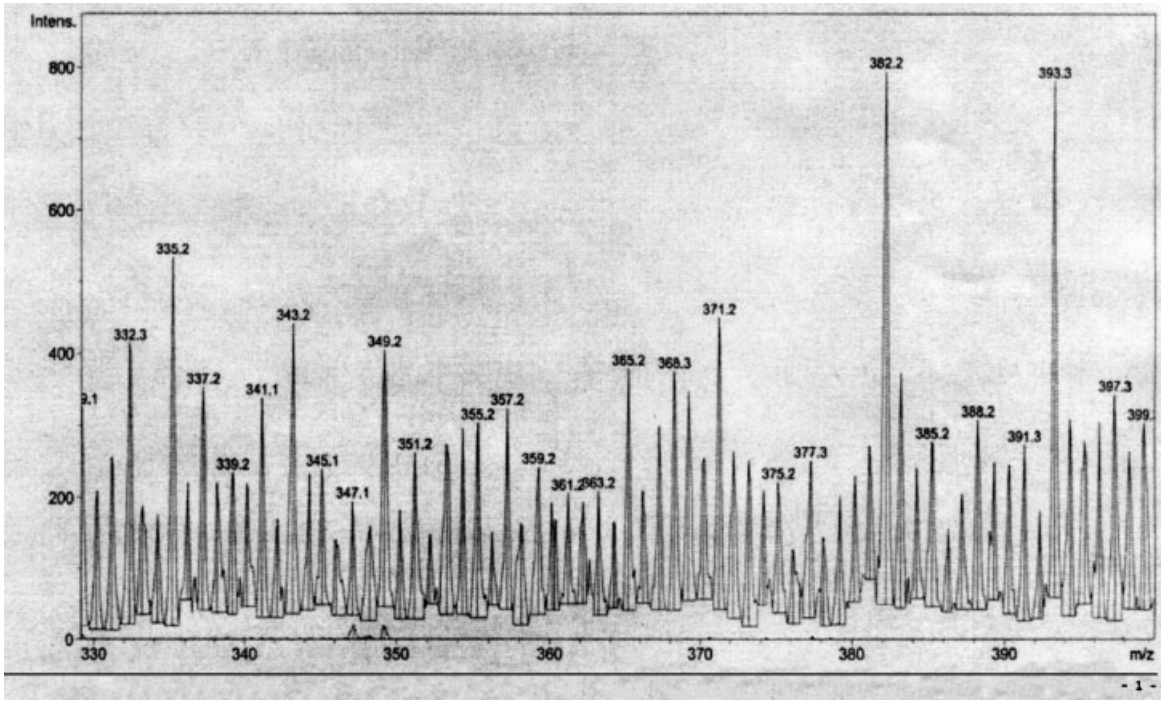

Fig. 3. Mass spectrometry analysis of epaulette shark fecal extract. Extraction was from pooled and concentrated elution peaks from 30 fecal extracts. The HPLC eluent was collected from minute 18.5 to minute 20.5 of a 45-minute elution profile. Molecular weight of corticosterone is 347. 1 (9^{th} numbered peak from left side of figure).

DISCUSSION

The present study has established the presence of the stress-related hormone, corticosterone, in the epaulette shark. It has also demonstrated that corticosterone can be reliably measured in fecal casts of this species. This finding may be useful for non-invasive monitoring of this elasmobranch, and perhaps other elasmobranch species that adapt well to captivity. The use of remotely-collected fecal casts avoids the stressful process of capture and blood collection, which has been the most common means of accessing non-benthic and/or wild elasmobranchs for endocrine assessment (Rasmussen and Crow, '93; Rasmussen and Gruber, '93; Snelson et al., '97). However, in captive elasmobranchs and possibly in wild shallow-benthic elasmobranchs targeted for studies aimed at assessment of chronic stress (i.e., requiring repetitive sampling over extended periods), collections of structurally sound fecal casts may be preferable to blood collection. The data from spiked samples, both tritiated and unlabeled, indicated good hormone recovery and low variability within both the extraction and the assay methodology. While the HPLC results using tritiated or exogenous, unlabeled corticosterone demonstrated that corticosterone was present in epaulette shark fecal extract, NMR spectrometry was required to demonstrate that the corticosterone was likely the only compound present in the eluent peak which contained corticosterone (19.6 min).

Although the stress response in elasmobranchs (Honn and Chavin, '78) appears in most ways similar to that in mammals (Nowell, '80; Asterita, '85), ACTH in elasmobranchs (not in mammals), causes the secretion of 1α-hydroxycorticosterone (Hazon and Henderson, '84). This is the principal corticosteroid involved in shark osmoregulation, and relatively small amounts of cortisol and corticosterone are produced (Idler and Truscott, '66; Honn and Chavin, '78; Klesch and Sage, '73; Hazon and Henderson, '84). The values of 1α-hydroxycorticosterone range from 0.08–13.9 μg/100 mL in the plasma of various elasmobranch species (Kime, '77). Fecal levels of this hormone are unknown.

While it is not the predominant corticoid in elasmobranchs, corticosterone may be a valid indicator of stress. However, its use as such is controversial. Corticosterone elevations have been demonstrated following physical stress in wild (Rasmussen and Hess, unpublished) and captive lemon sharks (Rasmussen and Schmidt, unpublished). On the other hand, Manire et al.

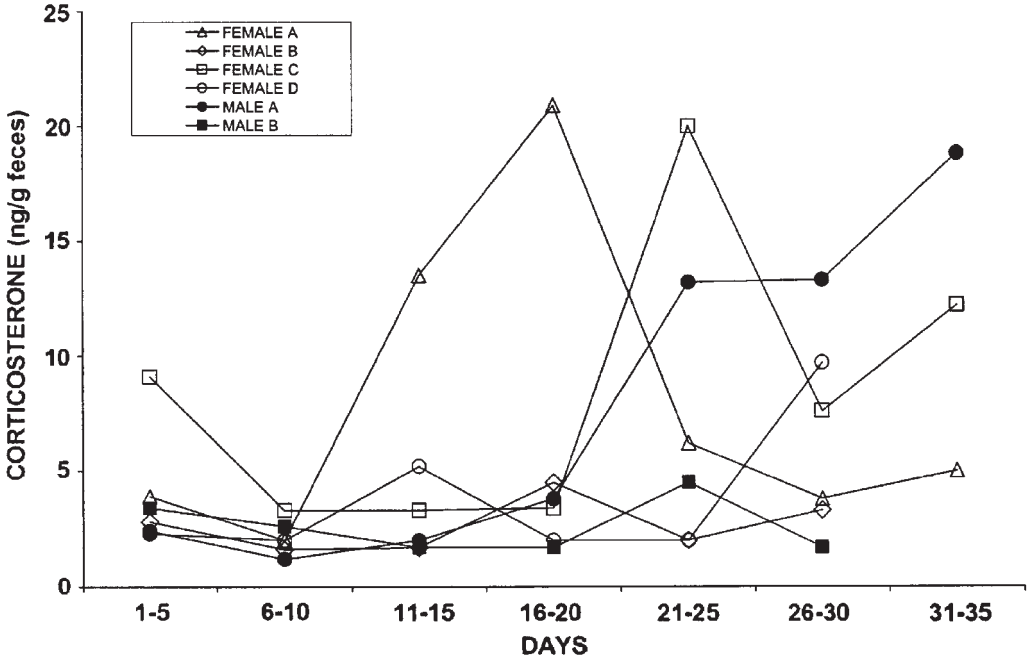

Fig. 4. Fecal corticosterone concentrations across time in individual undisturbed, captivity-raised epaulette sharks. Each data point is the average of a pool of samples from one shark collected across 5 consecutive days.

(unpublished) suggest that corticosterone levels are unrelated to stress in bonnethead sharks and Atlantic stingrays from the wild. Whether corticosterone is an indication of stress in the epaulette shark remains unknown. However, this study demonstrates the use of fecal casts in the detection of corticosterone, as long as the interference of 1α-hydroxycorticosterone can be ruled out. The fractionation-pattern data in Figure 2B for the fecal-extract eluent (30–sample pool) revealed both the absence of a peak at 363.1 (molecular weight of 1α-hydroxycorticosterone) and the presence of a peak at 329.1 (consistent with corticosterone but not 1α-hydroxycorticosterone). It is thus likely on the basis of these findings that the presumptive corticosterone elution peak (19.6 min) did contain corticosterone and did not contain measurable 1α-hydroxycorticosterone. The fact that corticosterone did not degrade during exposure to the fecal environment at room temperature for up to 12 hours after sample collection allows for some flexibility in collection and handling regimes for samples. For example, close monitoring of individuals for defecation is unnecessary, and use of overnight samples should be acceptable. In captive settings the use of non-invasive fecal samples collected without restraint or sedation has obvious advantages for stress-hormone monitoring. When the present study was undertaken, immunoassay for fecal corticoids was unavailable. HPLC was thus chosen for hormone measurement. In a small shark such as the epaulette, multiple samples are necessary to obtain sufficient hormone for HPLC-based hormone measurement. While this precludes point-in-time assessment of corticosterone status, it may be advantageous for assessing average state over time. In terms of future fecal corticosterone assessment in sharks, HPLC is generally less sensitive and less efficient than immunoassay. While the latter has been validated for fecal corticosterone measurement in some mammals, it has not been validated for fish or elasmobranch feces. On the basis of the present study, it appears that development of such an immunoassay would allow for a more sensitive measurement of stress hormones. The sensitive radio-immunoassay method for fecal glucocorticoids reported for mammals and birds (Wasser et al., 2000) may offer a reasonable starting point for the shark.

Despite the small n in this study, the difference between average across-time hormone values among individuals was less than within-individual hormone variation. Among the six sharks studied, the average difference between lowest and highest values across time was seven-fold. Four sharks exhibited a less than six-fold range and the remaining two sharks showed a 10.4–fold and a

15.6–fold range in this regard. Since the fecal hormone response to stress was not addressed in this study, it is not known whether the baseline hormone variability observed could mask the detection of a corticosterone response to stress. However, fecal corticoids in mammals have been shown to increase more than 25–fold in response to ACTH challenge in some species (Wasser et al., 2000), and average 10–fold increases in response to restraint and translocation have been reported (Palme et al., 2000; Turner et al., 2002). While it is possible that the sharks did not adapt fully to the laboratory condition, they had been captive-raised and showed no visible signs of stress during the study. Thus, it seems reasonable that the hormone levels obtained (Fig. 4) represented a minimal-stress or unstressed condition. On the basis of the results of the present study, further studies to determine possible fecal corticosterone response to stress in sharks appear warranted.

This is the first report of corticosterone presence in epaulette sharks and the first use of feces to measure corticosterone in an elasmobranch. It offers the possibility for assessing the status of stress-related steroid hormones in captive elasmobranchs by non-invasive means without handling. It is not known whether fecal hormone measurement will have application in free-ranging benthic elasmobranchs. For non-benthic deep-water elasmobranchs, access to fecal samples is unlikely.

In summary, this study has shown that remote collection of intact fecal casts from epaulette sharks is feasible, and that corticosterone can be measured in these feces using organic extraction and HPLC analysis. This study also provides baseline data for fecal corticosterone levels in six individual sharks across 33 days.

ACKNOWLEDGEMENTS

We wish to thank Jay Hemdal (curator, Toledo Zoo Aquarium) and Karla Jeselson (Florida Aquarium) for assistance in obtaining the sharks and consulting in their care and maintenance; J. Gano and Y. Kim for mass spectrometry and its interpretation.

LITERATURE CITED

Abelson A, Steinman BMF, Kaganovsky S. 1999. Mass transport from pollution sources to remote coral reefs in Eilat (Gulf of Aqaba, Red Sea). Mar Pollut Bull 38:25–29.

Asterita MF. 1985. The Physiology of Stress. Human Sciences Press, New York.

Barton BA, Iwama GK. 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annu Rev Fish Dis 1:3–26.

Bassett JM, Hinks NT. 1969. Micro-determination of corticosteroids in ovine peripheral plasma: effects of venipuncture, corticotrophin, insulin, and glucose. J Endocrinol 44:387–403.

Bonga L, Wendelaar SE. 1997. The stress response in fish. Physiol Rev 77:592–625.

Carpenter SR, Caraco NF, Correll DL, Howarth RW, Sharpley AN, Smith VH. 1998. Nonpoint pollution of surface waters with phosphorus and nitrogen. Ecol Appl 8:559–568.

Covey T. 1996. Liquid chromatography/mass spectrometry for the analysis of protein digests. Methods Mol Biol 61:83–99.

Dingerkus G. 1991. Sharks on the Great Barrier Reef. Tropical Fish Hobbyist 40:52–76.

Dingerkus G, DeFino T. 1983. A revision of Orectolobiform shark family Hemiscyllidae (*Chondrichthyes, Selachii*). Bull Am Mus Nat Hist 176:1–93.

Goymann W, Mostl E, Van't Hof T, East ML, Hofer H. 1999. Noninvasive fecal monitoring of glucocorticoids in spotted hyenas, *Crocuta crocuta*. Gen Comp Endocrinol 144: 340–348.

Grutter AS, Pankhurst NW. 2000. The effects of capture, handling, confinement, and ectoparasite load on plasma levels of cortisol, glucose, and lactate in the coral reef fish *Hemigymnus melapterus*. J Fish Biol 57:391–401.

Hazon N, Henderson IW. 1984. Secretory dynamics of 1α-hydroxycorticosterone in the elasmobranch fish, *Scyliorhinus canicula*. J Endocrinol 103:205–211.

Heupel MR, Bennett MB. 1998. Observations on the diet and feeding habits of the Epaulette Shark, Hemiscyllium ocellatum (*Bonnaterre*), on Heron Island Reef, Great Barrier Reef, Australia. Marine and Freshwater Research 49: 753–756.

Heupel MR, Whittier JM, Bennett MB. 1999. Plasma steroid hormone profiles and reproductive biology of the epaulette shark, *Hemiscyllium ocellatum*. J Exp Zool 284: 586–594.

Honn KV, Chavin W. 1978. In vitro tropic action of ACTH upon adrenocortical cyclic nucleotide (cAMP, cGMP) production and corticosterone output in blue (*Prionace glauca Linnaeus*) and in mako (*Isurus oxyrinchus Rafinesque*) sharks. Gen Comp Endocrinol 36:161–169.

Idler DR, Truscott B. 1966. 1 α-Hydroxycorticosterone from cartilaginous fish: a new adrenal steroid in blood. Journal of the Fisheries Research Board of Canada 23:615–619.

Jurke MH, Czekala NM, Lindburg DG, Millard SE. 1997. Fecal corticoid metabolite measurement in the cheetah (*Acinonyx jubatus*). Zoo Biol 16:113–147.

Kime DE. 1977. Measurement of 1alpha-hydroxycorticosterone and other corticosteroids in elasmobranch plasma by radioimmunoassay. Gen Comp Endocrinol 33: 344–351.

Klesch WL, Sage M. 1973. The control of the interrenal by the pituitary in the elasmobranch, Dasyatis sabina. Comp Biochem Physiol A Physiol 45:961–967.

Last PR, Stevens J.D. 1994. Sharks and Rays of Australia. CSIRO, Australia.

Miller MW, Hobbs NT, Sousa MC. 1991. Detecting stress responses in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*): reliability of cortisol concentrations in urine and feces. Can J Zool 69:16–25.

Nowell NW. 1980. Adrenocortical function in relation to mammalian population densities and hierarchies. In: Chester-Jones I, Henderson IW, editors. General, Comparative and Clinical Endocrinology of the Adrenal Cortex. London: Academic Press.

Palme R, Robia C, Baumgartner W, Mostl E. 2000. Transport stress in cattle as reflected by an increase in fecal cortisol metabolite concentrations. Vet Rec 146: 108–109.

Rasmussen LEL, Crow GL. 1993. A three-year study of serum corticosterone levels in juvenile reef whitetip sharks (*Triaenodon obesus*). J Exp Zool 267:283–287.

Rasmussen LEL, Gruber SH. 1993. Serum levels of reproductively-related circulating steroid hormones in the free-ranging lemon shark, *Negaprion brevirostris*. Environmental Biology 38:167–174.

Rogers CS, Garrison G, Grober R, Hills Z, Franke M. 1994. Coral Reef Monitoring Manual for the Caribbean and Western Atlantic. Atlanta, GA: National Park Service Publication.

Snelson FF, Jr, Rasmussen LE, Johnson MR, Hess DL. 1997. Serum concentrations of steroid hormones during reproduction in the Atlantic stingray, *Dasyatis sabina*. Gen Comp Endocrinol 108:67–79.

Stephens DB. 1980. Stress and its measurement in domestic animals: a review of behavioral and physiological studies under field and laboratory situations. Adv Vet Sci Comp Med 24:179–210.

Turner JW, Jr, Tolson P, Hamad N. 2002. Remote assessment of stress in white rhinoceros (*Certotherium simum*) and black rhinoceros (*Dicerosbicornis*) via measurement of adrenal steroid in feces. J Zoo Wildl Med 33:214–221.

Wasser SK, Hunt KE, Brown JL, Cooper K, Crockett CM, Bechert U, Millspaugh JJ, Larson S, Monfort SL. 2000. A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. Gen Comp Endocrinol 120:260–275.

Wasser SK, Starling AK. 1988. Proximate and ultimate causes of reproductive suppression among female yellow baboons at Mikumi National Park, Tanzania. Am J Primatol 16:97–121.

Whitten PL. 1997. Noninvasive methods for the study of stress, reproductive function, and aggression. Am J Phys Anthropol 24:239.