

## Measurement of fecal glucocorticoids in parrotfishes to assess stress.

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Coral reefs are in decline worldwide from a combination of natural and human forces. The environmental compromises faced by coral reef habitats and their associated fishes are potentially stressful, and in this study we examined the potential for assessing stress levels in coral reef fish. We determined the feasibility of using fecal casts from parrotfishes for remote assessment of stress-related hormones (cortisol and corticosterone), and the response of these hormones to the stress of restraint and hypoxia. Measurement of these hormones in fecal extracts by high performance liquid chromatography (HPLC) was validated using mass spectrometry, chemical derivitization, and radioactive tracer methods. In aquarium-adapted parrotfish, baseline levels of cortisol and corticosterone averaged  $3.4 \pm 1.1$  and  $14.8 \pm 2.8$  ng/g feces, respectively, across 32 days. During 13 days of periodic stress these hormones, respectively, average 10.8-fold and 3.2-fold greater than baseline, with a return to near baseline during a 23-day follow-up. Testosterone was also measured as a reference hormone which is not part of the stress-response axis. Levels of this hormone were similar across the study. These fecal hormones were also measured in a field study of parrotfish in 10 fringing coral reef areas around the Caribbean Island of St. John, US Virgin Islands. Extracts of remotely collected fecal casts of three parrotfish species revealed no difference in respective average hormone levels among these species. Also, there was no difference in respective hormone levels between aquarium and field environments. However, levels of both cortisol and corticosterone, but not testosterone, were elevated in two of the 10 reef sites surveyed. This study demonstrates that parrotfish fecals can be collected in aquarium and field conditions and that steroid hormones in these fecals can be extracted and reliably measured. The study also demonstrates that cortisol and corticosterone in parrotfish fecals can be used as an indicator of the stress-response which is unlikely to be masked by intrinsic variability in the sample source, environment or methodology.

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## Measurement of fecal glucocorticoids in parrotfishes to assess stress

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### Abstract

Coral reefs are in decline worldwide from a combination of natural and human forces. The environmental compromises faced by coral reef habitats and their associated fishes are potentially stressful, and in this study we examined the potential for assessing stress levels in coral reef fish. We determined the feasibility of using fecal casts from parrotfishes for remote assessment of stress-related hormones (cortisol and corticosterone), and the response of these hormones to the stress of restraint and hypoxia. Measurement of these hormones in fecal extracts by high performance liquid chromatography (HPLC) was validated using mass spectrometry, chemical derivitization, and radioactive tracer methods. In aquarium-adapted parrotfish, baseline levels of cortisol and corticosterone averaged  $3.4 \pm 1.1$  and  $14.8 \pm 2.8$  ng/g feces, respectively, across 32 days. During 13 days of periodic stress these hormones, respectively, average 10.8-fold and 3.2-fold greater than baseline, with a return to near baseline during a 23-day follow-up. Testosterone was also measured as a reference hormone which is not part of the stress response axis. Levels of this hormone were similar across the study. These fecal hormones were also measured in a field study of parrotfish in 10 fringing coral reef areas around the Caribbean Island of St. John, US Virgin Islands. Extracts of remotely collected fecal casts of three parrotfish species revealed no difference in respective average hormone levels among these species. Also, there was no difference in respective hormone levels between aquarium and field environments. However, levels of both cortisol and corticosterone, but not testosterone, were elevated in two of the 10 reef sites surveyed. This study demonstrates that parrotfish fecals can be collected in aquarium and field conditions and that steroid hormones in these fecals can be extracted and reliably measured. The study also demonstrates that cortisol and corticosterone in parrotfish fecals can be used as an indicator of the stress response which is unlikely to be masked by intrinsic variability in the sample source, environment or methodology.

**Author Keywords:** Fecal glucocorticoids; Parrotfishes; Stress

### Article Outline

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- 1. Introduction
- 2. Materials and methods
  - 2.1. Experimental design
  - 2.2. Aquarium study
    - 2.2.1. Fish husbandry
    - 2.2.2. Aquarium environment
    - 2.2.3. Fecal and blood collection
    - 2.2.4. Stress protocols
  - 2.3. Sample extraction and reconstitution
  - 2.4. HPLC analysis
  - 2.5. Verification of hormone identity
  - 2.6. Field study
    - 2.6.1. Study site
    - 2.6.2. Sample collection protocol
  - 2.7. Data analysis
- 3. Results
  - 3.1. Aquarium study
    - 3.1.1. General
    - 3.1.2. Stress study
  - 3.2. Field study
- 4. Discussion
  - 4.1. Methodological
  - 4.2. Aquarium
  - 4.3. Field
- Acknowledgements
- References

## 1. Introduction

Endocrine activity in a variety of species can be markedly altered under stressful conditions ([Asterita, 1985]). Specifically, both acute and chronic stressors of various types have consistently resulted in significantly increased secretion of steroid hormones from the adrenal gland in mammals ([Mulrow, 1986]) and extrarenal gland in fish ([Redding, 1993]; [Schreck, 1990]); reviewed ([Iwama et al., 1997]). Responses to acute and chronic stresses have been quantified using blood levels of the glucocorticoids cortisol or corticosterone in numerous species, including freshwater and marine fish ([Barton et al., 2000]; [Barton and Iwama, 1991]; [Bonga and Wendelaar, 1997]; [Campbell et al., 1992]; [Sunyer et al., 1995]).

Steroid hormone measurement in urine and feces originally focused on reproductive steroids (reviewed, [Lasley and Kirkpatrick, 1991]). A major impetus for this approach was the ability to access endocrine data from dangerous captive exotic species ([Safer-Hermann et al., 1987]) and free-roaming wildlife ([Kirkpatrick et al., 1990]) without immobilization and handling. In addition to offering reasonable access to target animals this approach has avoided the possible confounding effects of stressful immobilization and blood sampling on levels of measured hormones ([Miller et al., 1991]). Also, fecal hormone measurement represents the average of hormone production across time and is thus less subject to the acute and potentially misleading hormone traverses which can be associated with point-in-time blood hormone measurement.

Fecal glucocorticoids have been used to assess response to stress in various mammals and birds ([Wasser et al., 2000]), including response to restraint in captive female cheetahs ([Jurke et al., 1997]), social stress in spotted hyenas ([Goymann et al., 1999]), translocation stress in rhinoceros ([Turner et al., 2002]), and captivity in chimpanzees ([Whitten, 1997]). Fecal glucocorticoid measurement has not been reported for fish.

Coral reefs are in decline worldwide ([Wilkinson, 2002]) from a combination of natural and human stressors. In light of many environmental compromises faced by coral reef habitats in recent years ([Bythell et al., 1993]; [Hixon and Beets, 1993]; [Hughes, 1994]; [Hunte, 1992]; [Richmond, 1993]; [Rogers, 1990]; [Rogers and Beets, 2001]; [Russ, 1991]; [Wolff et al., 1999]), it may be useful to determine stress hormone levels in coral reef fish using non-invasive methods.

The objectives of the present study were (1) to develop a method for assessment of fecal glucocorticoids (specifically cortisol and corticosterone) as a measure of stress in coral reef fishes, specifically parrotfishes, and (2) to determine feasibility of applying this methodology to these fishes in a field setting. We hypothesize that (1) deposited fecal casts can be readily obtained in both aquarium and varied field settings, (2) these fecal casts can be used for routine extraction and reliable measurement of cortisol and corticosterone, (3) fecal cortisol and corticosterone levels will increase in response to hypoxia/restraint stress, and (4) fecal cortisol and corticosterone can serve as a measure of stress in the field.

## 2. Materials and methods

### 2.1. Experimental design

These studies were performed under the auspices of animal-use protocol (IACUC #100679) at the Medical College of Ohio. Prior to beginning the protocol, several genera were screened for potential experimental use. Selection was based on their worldwide association with coral reefs, obligation to coral reef habitat (thus potentially reflective of habitat status), presence in shallow (<10 m) water (facilitating collection), and fecal productivity (volume and frequency). In order of descending acceptability these genera were: parrotfishes (*Scarus* sp. and *Sparisoma* sp.), grouper (*Epinephelus* sp.), chub (*Kyphosus* sp.), puffer (*Diodon* sp.), porkfish (*Anisotremus* sp.), grunt (*Haemulon* sp.), doctor fish (*Acanthurus* sp.) and angelfish (*Holocanthus* sp.). Parrotfishes satisfied all criteria and showed several-fold greater fecal productivity and frequency than any other species examined. They were therefore chosen for the study. The experimental animals were stoplight, queen and rainbow parrotfish (*Sparisoma viride*, *Scarus vetula*, and *Scarus guacamaia*, respectively), obtained from a commercial supplier (aquarium study) or located on site on fringing reefs around St. John, US Virgin Islands.

The aquarium component of these studies focused on feasibility and reliability of hormone extraction and measurement and on the hormone response to the stress of repeated hypoxia and restraint. The experiments employed a repeated-measures design of baseline, treatment and follow-up segments ([Zar, 1984]). The field component involved collection of fresh fecal casts in a natural setting and comparison of average hormone levels among fish inhabiting different bays. It also permitted preliminary fecal hormone-level comparison between fish adapted to captivity (aquarium) versus fish free-swimming on the reef.

In both aquarium and field experiments the measurements were made from pooled daily samples, since insufficient fecal material was available from one fish fecal cast for assured analysis. The use of pooled samples was consistent with the goals of the study, in that eventual application of this methodology would likely involve monitoring average stress levels across time.

### 2.2. Aquarium study

#### 2.2.1. Fish husbandry

Species were obtained from commercial suppliers and quarantined in copper-treated (<0.2 ppm) artificial seawater for 2 weeks to eliminate parasites. Three weeks prior to the onset of the baseline period, they were transferred to the study aquarium for acclimation. Diet consisted of pellets of seaweed, smelt and koi food sticks ground and suspended in gelatin cut into small cubes.

The large size of adult parrotfish, often >50 cm length precluded the use of adults in the aquarium study. Thus, experimental aquarium subjects were six juvenile rainbow parrotfish (*S. guacamaia*), sex unknown, ranging from 25 to 35 cm. All fish appeared well-adapted, were eating well prior to the study and interacted peacefully throughout the study. One fish died of undetermined causes 1.5 months after completion of the study. Another fish died of undetermined causes 3 months later.

#### 2.2.2. Aquarium environment

Fish were kept in a rectangular aquarium system containing 1300 liters of artificial seawater (Instant Ocean, Aquarium Systems, Mentor, OH). Light was provided on a 12-h light/dark cycle using spectral fluorescent tubes (105 W total). Water quality was assured by regular 10% water changes, physical/biological filters, and automated protein skimmers. A plastic grate (approximately 2.5 cm mesh) was placed across the entire aquarium floor, 8 cm above the bottom, to prevent fish from disturbing settled feces. Water quality was checked twice weekly, and criteria were: specific gravity (1.018–1.023), pH (7.80–8.20) and temperature (25–28 °C), i.e., similar to natural environment. Ammonia levels were checked weekly and maintained below 0.2 ppm by water change when necessary (rarely). The fish were fed daily between 9 AM and 11 AM.

#### 2.2.3. Fecal and blood collection

Feces were produced as solid casts, which sank to the bottom and were retrieved by siphon tube. Collections were made twice daily (9 AM and 5 PM)

to minimize fecal dispersal on the aquarium bottom and possible hormone degradation across time at aquarium temperature. Daily collections were pooled across fish prior to storage, providing a single average daily aquarium value for each hormone measured. Neither degree of sample dispersal nor water volume of the sample influenced the hormone content of the fecal material, as determined by the analysis of aliquots of (1) intact vs. dispersed fecal casts and (2) pooled fecal sample containing water by volume from a 1:1 ratio up to a 5:1 ratio. Collected, pooled samples were allowed to settle in the vial, which was frozen at 40 °C until extracted.

Since blood levels of cortisol and corticosterone have not been previously reported for parrotfish, blood was collected for plasma hormone measurement and to permit fecal/plasma hormone comparison. Approximately one month after completion of the stress response study, individual parrotfish were separated from each other by plastic mesh dividers. This permitted collection of fecals from individual fish. After 10 days of acclimation each fish was separately removed from the aquarium and placed in a bucket of seawater containing the sedative tricaine methanesulfonate (MS-222, Argent Chemical Labs, Redmond, WA) at a concentration of 70 mg/liter. Upon sedation (<4 min) each fish was removed from the bucket, blood-sampled (up to 1.0 cc) via tail vein ([Stoskopf, 1993]) and returned to the aquarium. Fecal samples for fecal vs. blood hormone comparison were collected from each of these fish for 4 days prior to blood collection. This procedure was repeated 10 days later, and respective samples were pooled with the first set to assure sufficient material for analysis. This procedure yielded one pooled fecal and one pooled blood sample for analysis from each of the six fish.

#### 2.2.4. Stress protocols

All parrotfish were exposed to a protocol consisting of a 32-day baseline, three repeated stress periods involving removal from the water (acute hypoxia) with handling (restraint) across 13 days and a 23-day follow-up period. Fish were fed daily throughout the protocol.

While restraint and severe hypoxia are unlikely to occur under natural conditions, they are well known to elicit a strong stress response. Thus, restraint/hypoxia was used in this study to maximize potential for observing a hormonal response to stress. We reasoned that, pending observation of a response in this study, more natural stressors could subsequently be examined. The stress protocol was derived from behavioral observations of response and recovery of parrotfish subjected to various temporal patterns of netting with removal from the water.

Specifically, the hypoxia/restraint stressor consisted of netting and removing each fish from the water for 30 s, three separate times across 30 min, beginning between 10 AM and noon. This stress procedure was repeated hourly five times, and the entire 5-h procedure was repeated the next day. This 2-day protocol was performed three times in 13 days (days 1, 2, 6, 7, and 12, 13 of the stress portion of the study). Fish were monitored for visible signs of stress, including rapid respiration, color changes, inactivity and unresponsiveness. These signs of stress disappeared within 2 h post-stress on a given day.

#### 2.3. Sample extraction and reconstitution

Fecal samples were extracted and reconstituted for simultaneous, cortisol, corticosterone and testosterone measurement. Corticosterone was included because it was present in fish sample elution profiles and is a steroid known to be associated with stress in other vertebrates, including many mammals, sharks, and reptiles. We felt that, in light of the ready availability of the corticosterone data, it could be a useful alternative measure of stress responding. The primary purpose of testosterone measurement was to use it as a reference hormone, since its response to acute stress is usually limited ([Mulrow, 1986]).

Eighteen ml of dichloromethane was added to the vial containing a 6 ml fecal slurry (1:1, water:solid), and shaken vigorously on a motorized shaker (Burrel, Pittsburgh, PA) for 1 h. The vial was then centrifuged at 2000g for 10 min to separate the fecal, water, and dichloromethane layers. The bottom (dichloromethane) layer (16 ml) was recovered and vortexed for 2 min with 1 ml of 0.1 N NaOH to remove any remaining water-soluble materials. The dichloromethane layer was recovered, mixed with 1 ml of 18 M water and vortexed for 2 min. The dichloromethane layer (15 ml) was then pipetted into borosilicate glass culture tubes and filtered (Pall Gelman Acrodisc 0.45 µm mesh membrane filters, Louisville, KY). The filtrate was evaporated to dryness in a vacuum centrifuge, and the samples were covered with parafilm and stored at 37 °C until reconstitution.

The dried samples were brought to room temperature, reconstituted in 100% HPLC-grade acetonitrile (ACN) and diluted with 18 M water to yield 500 µl of a 10% ACN solution. A 250 µl aliquot of the clear reconstitute was then analyzed by High Performance Liquid Chromatography (HPLC) for cortisol, corticosterone, and testosterone.

#### 2.4. HPLC analysis

HPLC was chosen for hormone measurement because it enabled measurement of all three hormones in a single extract. Also, a reliable fecal radioimmunoassay such as is currently in use for fecal glucocorticoids ([Wasser et al., 2000]), was not available at the time of these analyses.

The hormone measurement system was a Reverse-Phase HPLC (Dionex, Sunnyvale, CA) employing a standard 3.9 × 300 mm, C-18 column (Waters, Milford, MA) and a variable wavelength UV detector set at 240 nm. Prior to sample analyses a water blank was run until the column was free of major peaks, and a reference standard containing cortisol, corticosterone, and testosterone was run to verify retention times. The flow rate was 1 ml/min, and the elution gradient changed from 10% ACN/90% water to 90% ACN/10% water over a period of 45 min, ensuring complete separation of sample compounds. The standard curve for each hormone was developed by HPLC runs of duplicate samples of 10 known concentrations of each hormone. The correlation coefficient for actual dilutions vs. the calculated logarithmic curve ( $y = a e^x$ ) averaged 0.995 for cortisol, 0.992 for corticosterone, and 0.989 for testosterone. Hormone values are reported as ng/g feces (wet wt.). The lower limit of hormone detection was 1.8 ng per sample. Hormone levels in extracts rarely approached this limit.

## 2.5. Verification of hormone identity

Because fecal extracts contain numerous compounds, it was necessary to verify that the HPLC peaks assigned as cortisol, corticosterone and testosterone contained these hormones and only these hormones. We made this verification by three methods: (1) chemical derivitization, (2) radioactive tracer, and (3) mass spectrometry. Although these HPLC verification methods have been previously reported ([Turner et al., 2002]), they are briefly described below.

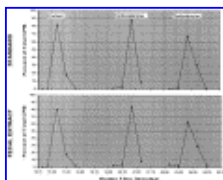
Derivitization was performed on cortisol, corticosterone and testosterone standards and on eluent peaks of presumptive cortisol, corticosterone and testosterone obtained from fecal extracts, using the derivitizing agent methoxyamine-HCl (MOX) in pyridine (Pierce Chemical, Rockford, IL). The derivitization converted the carbonyl group at the 3 and 20 positions in the steroids to a methoxime group at these positions, resulting in a characteristic shift in HPLC elution time. To determine the derivitization response of presumptive hormone eluent peaks from fecal extracts, for each hormone a pool of eluent peaks was prepared from HPLC analyses of 15 separate fecal extracts. Each of the three pools of peaks (one pool for each hormone) was then subjected to derivitization and injected into the HPLC. Derivitization data are presented in Table 1.

Table 1. Effect of chemical derivitization on HPLC elution times for cortisol, corticosterone, and testosterone

Sample type	Elution time (min) <sup>b</sup>		
	Cortisol	Corticosterone	Testosterone
Underderivitized standard	16.64	19.85	23.88
Derivitized standard	28.11	33.91	37.07
Derivitized fecal extract <sup>c</sup>	27.95	33.80	36.88

Eluent peaks for cortisol and corticosterone standards and presumptive cortisol and corticosterone eluent peaks from fecal extracts were collected and sent to J. Gano (University of Toledo, Toledo, OH) for mass spectrometry analysis. Testosterone was not analyzed. The spectrometric fractionation patterns of the cortisol and corticosterone standard eluents revealed molecular weights of 362.5 and 347.3, respectively. A pool of 15 eluent peaks presumptive for cortisol yielded a molecular weight of 362.2 and a similar 15-peak pool presumptive for corticosterone yielded a molecular weight of 347.1. The fractionation pattern for cortisol standard contained characteristic, distinct peaks at an *m/z* (ion mass:charge ratio) of 344, 333, and 302, with each peak representing a different ionic fragment of the molecule. Two of these peaks (344 and 302) were detectable in the fecal eluent pool. Fractionation of corticosterone standard yielded identifying peaks at 329, 311, and 293, and the fecal eluent produced peaks at 329 and 311, but no peak at 293. The lowest of the three peaks for each standard was the absent peak in each fecal eluent, likely reflecting insufficient hormone in these eluents for detection of those peaks. The peaks which were observed were considered sufficient to identify the compounds (J. Gano, personal communication). These data indicate that the fecal extracts contained cortisol and corticosterone, respectively, and were likely not contaminated by other compounds.

Final assessment of hormone identity in HPLC eluents was made by spiking separate fecal extracts with tritiated cortisol or corticosterone or testosterone (ICN Pharmaceuticals, Costa Mesa, CA) and determining radioactivity in HPLC eluents from these fecal extracts by liquid scintillation. Only eluent peaks coincident with the respective retention times for these hormones showed significant radioactivity (Fig. 1) and the recoveries of added label were 91.8 (cortisol), 89.8 (corticosterone), and 90.5 (testosterone) percent.



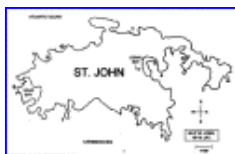
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Fig. 1. HPLC eluent profiles of tritiated steroid hormones: comparison of hormone standards against fecal extracts. Each hormone was determined in a separate extract to avoid radioactivity cross contamination. Total CPM added per sample was 72,411 (cortisol), 54,829 (corticosterone), and 61,030 (testosterone).

## 2.6. Field study

### 2.6.1. Study site

In February 1999 fecal samples were collected from parrotfishes inhabiting fringing coral reefs in 10 different bays (or off the rocky points delineating these bays) distributed around St. John, US Virgin Islands (Fig. 2). Virgin Islands National Park comprises 56% of the island (18°N. latitude and 64.5° W. longitude) and includes about one half of more than 35 bays fringing the island. Six of the 10 bays in the study were within the Park. Sample collection sites were chosen by the following criteria: (1) presence of adult parrotfish (*S. viride* and *S. vetula*), (2) reef <50 m off the shore, (3) minimum reef area of 300 m<sup>2</sup> at 3 . 10 m depth, and (4) subjectively similar reef characteristics in terms of physical relief and coral cover. Several physical characteristics of the chosen reef sites were measured to enable more objective site comparisons (Table 2). Methods used for these measurements have been previously reported ([Rogers et al., 1994]). Repeated collections focused on the same portion of each reef site each time, using specific natural objects on the reef as reference markers.



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Fig. 2. Diagram of the island of St. John, US Virgin Islands, showing parrotfish fecal-collection sites. Cruz Bay and Coral Bay are towns and have the greatest population density. Numbered sites on the map are named (from National Park Service) as follows: 1, Leinster Bay (LN); 2, Haulover Bay (HL); 3, Long Point (LP); 4, Harbor Point (HP); 5, Saltpond Bay (SL); 6, Great Lameshur Bay (LM); 7, Rendezvous Bay (RN); 8, Gallows Point (GP); 9, Hawksnest Bay (HK); and 10, Trunk Bay (TR). Sites not in the Virgin Islands National Park are 3, 4, 7, and 8.

Table 2. Site characteristics of 10 fringing reef areas on St. Johnes utilized by parrotfishes

Site	Visibility (m) <sup>b</sup>	Turbidity (NTU) <sup>c</sup>	Average reef depth (m) <sup>d</sup>	Reef rugosity <sup>e</sup>	% Live coral <sup>f</sup>	Habitat type <sup>g</sup>
Leinster Bay	10.1	0.92	3	0.28	22	SB
Haulover Bay	8.5	0.91	4	0.39	28	SB
Long Point	8.0	0.94	4	0.61	22	RK
Harbor Point	6.1	1.38	3	0.43	20	RK
Saltpond Bay	12.2	0.83	6	0.50	24	SB/RK
Great Lameshur Bay	10.6	1.04	5	0.42	28	SB/RK
Rendezvous Bay	8.0	.90	6	0.55	22	RK
Gallows Point	8.1	1.09	4	0.52	18	SB/RK
Hawksnest Bay	6.0	1.15	3	0.40	16	SB
Trunk Bay	5.0	1.01	3	0.31	14	SB/RK

### 2.6.2. Sample collection protocol

Sites were accessed using SCUBA or snorkeling, and samples were collected by following parrotfishes until they defecated. Parrotfishes are not generally skittish. We attempted to minimize stress to them by following at distances at which they continued routine activities and seemed undisturbed by our presence. If a given fish was stressed by our presence, it is unlikely that hormonal response to this would appear in samples collected in that session, since GI transit times in fish are generally longer than the 15 . 30 min intervals between defecations in parrotfishes. Intact samples were readily collected when fish were moving slowly and <1 m above the reef/seabed. Individual fecal casts were usually of insufficient volume for assay. Therefore, casts from six fish (three male, three female) were collected into a single, 20 cc polypropylene vial. The pooled vial content was extracted and assayed as a unit, yielding a single data point (i.e., six fish per single hormone value).















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