ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

General and Comparative Endocrinology 133 (2003) 341–352

GENERAL AND COMPARATIVE ENDOCRINOLOGY

www.elsevier.com/locate/ygcen

Measurement of fecal glucocorticoids in parrotfishes to assess stress

John W. Turner Jr.,[a,*] Richard Nemeth,[b] and Caroline Rogers[c]

[a] *Department of Physiology and Molecular Medicine, Medical College of Ohio, Toledo, OH 43614-5804, USA*
[b] *Center for Marine and Environmental Studies, University of the Virgin Islands Charlotte Amalie, St. Thomas, US Virgin Islands 00802, USA*
[c] *US Department of Interior, US Geological Survey, Caribbean Field Station St. John, US Virgin Islands 00830, USA*

Accepted 12 May 2003

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 ± 1.1 and 14.8 ± 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.

Keywords: Fecal glucocorticoids; Parrotfishes; Stress

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,

* Corresponding author. Fax: 1-419-383-6168.
E-mail address: jturner@mco.edu (J.W. Turner Jr.).

0016-6480/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0016-6480(03)00196-5

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) artifical 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 artifical 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 2000*g* 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 MΩ 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 MΩ 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 ηm. 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 derivatizing 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.

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

Table 1
Effect of chemical deriviation[a] on HPLC elution times for cortisol, corticosterone, and testosterone

Sample type	Elution time (min)[b]		
	Cortisol	Corticosterone	Testosterone
Underivitized standard	16.64	19.85	23.88
Derivitized standard	28.11	33.91	37.07
Derivitized fecal extract[c]	27.95	33.80	36.88

[a] Derivitization utilized methoxyamine-HCl to chemically alter each steroid, yielding a characteristic shift in time of the elution peak for each steroid.

[b] Time from injection of sample to highest point of elution peak.

[c] For each hormone, an HPLC eluent peak presumptive for that hormone was obtained from 15 separate fecal extracts. These 15 eluent peaks were pooled, and the pool was derivatized, i.e., a separate eluent pool was prepared for each hormone, derivitized and analyzed on HPLC.

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.

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^2 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.

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).

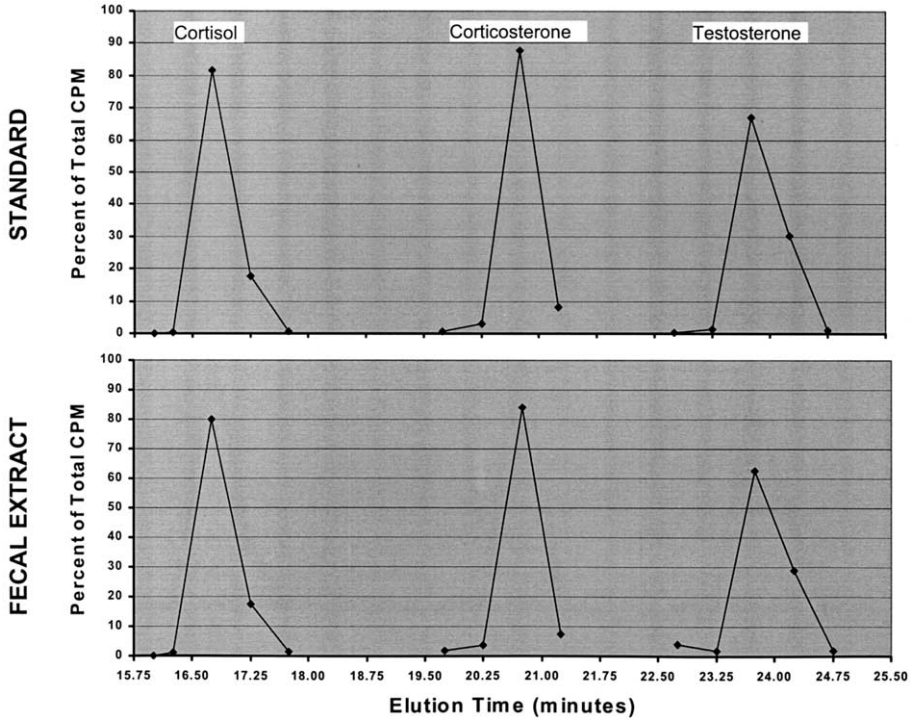

Fig. 1. HPLC eluent profiles of tritiated steroid hormones: comparison of hormone standards against fecal extracts. Each hormone was determined in a separate extract to avoid radioactivity cross contamination. Total CPM added per sample was 72,411 (cortisol), 54,829 (corticosterone), and 61,030 (testosterone).

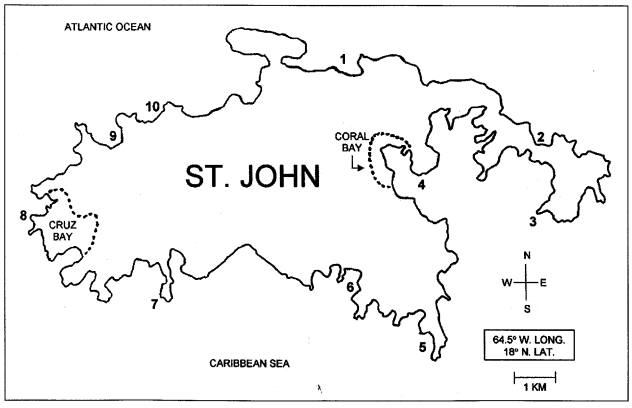

Fig. 2. Diagram of the island of St. John, US Virgin Islands, showing parrotfish fecal-collection sites. Cruz Bay and Coral Bay are towns and have the greatest population density. Numbered sites on the map are named (from National Park Service) as follows: 1, Leinster Bay (LN); 2, Haulover Bay (HL); 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.

Samples from the two different species were kept separate initially. However, no difference was observed in average hormone levels between species in these samples (Table 3), so samples from either species were collected into a single vial for the remainder of the study to maximize collection efficiency. Each bay was sampled three times over a 3-week period. Samples were transported to shore at ambient temperature and were frozen until extracted and assayed. Prior to extraction, samples were thawed, mixed and allowed to settle for 10 min. This yielded separation of the fecal layer from sand and water. Three grams of settled feces were used for extraction.

In order to compare field and aquarium hormone levels, fecal collections from juvenile rainbow parrotfish were attempted. However, the smaller volume of juvenile fecals and the lower density of this species relative to queen and stoplight parrotfish at most field sites precluded routine collections from juvenile rainbow parrotfish. In February 2000 sufficient fecal collections were made from juvenile Rainbow parrotfish at one field site

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

Site	Visibility (m)[b]	Turbidity (NTU)[c]	Average reef depth (m)[d]	Reef rugosity[e]	% Live coral[f]	Habitat type[g]
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

[a] Characteristics are the average of three samples across 3 weeks.
[b] Length of string to disappearance of secchi disc at end of string.
[c] Light scattering (Nephelometer turbidity units, NTU) of water samples obtained midway between surface and bottom. Each of the three sample days was the average of two samples taken 5 m apart at same depth.
[d] Depth below surface midway between top and bottom of reef.
[e] Fractional measure of reef relief: length of a chain (denominator) dropped over the reef contours across three linear, horizontal meters (numerator).
[f] Percent of a given 5 m horizontal length of reef which is comprised of live coral (average of three locations measured per site).
[g] Habitat types were coral on sand bottom (SB), rock (RK) or a mix of sand bottom and rock (SB/RK).

Table 3
Steroid hormone levels in remotely collected fresh fecal casts from adult parrotfishes occupying fringing coral reef on St. John, US Virgin Islands

Parrotfish type[a]	n[b]	Fecal hormone level ng/g feces[c]		
		Cortisol	Corticosterone	Testosterone
Queen (*Scarus vetula*)	5	8.8 ± 3.5	18.0 ± 4.1	6.6 ± 2.7
Stoplight (*Sparisoma viride*)	5	6.1 ± 1.5	12.9 ± 2.8	10.3 ± 4.7

[a] All fish were adults, free-swimming on the reef.
[b] Each collection vial contained six fecal casts, three from males and three from females. The n refers to the number of pooled samples tested to yield an average value. The contents of each vial were hormone-extracted as a single, pooled sample.
[c] No differences between species for any hormone, Student's t test, $p > 0.05$.

(Great Lameshur Bay) to permit hormone measurement for field vs. aquarium comparison. Collections and sample handling were the same as for the other species, except that the three vials contained samples from nine fish rather than six fish. As in the aquarium study, the sex of these juvenile fish could not be determined.

2.7. Data analysis

Data are presented where appropriate as mean value ($\bar{x}$) ± standard error (SE) in units of nanograms (ng) per gram of feces, except for blood data, which are presented as nanograms (ng) per ml of plasma. Zar (1984) was the reference for all statistical testing, and probability of $p \leqslant 0.05$ was considered significant for rejection of the null hypothesis. In cases where two groups or conditions were compared, Student's *t* test was used. Hormone data in the stress experiment were analyzed statistically by averaging values obtained for each period (baseline, stress, and follow-up) and subjecting them to Repeated Measures ANOVA and Paired-Sample *t* test. In the field study average hormone values were determined by treating each pooled sample as a single data point and averaging sets of data points for each site. These data were assessed by ANOVA and Tukey test. Data for site characteristics were analyzed by non-parametric Kruskal–Wallis test (for a given characteristic across sites). Pearson correlation was used to assess possible correlation across sites between a given site characteristic and cortisol or corticosterone levels.

3. Results

3.1. Aquarium study

3.1.1. General

All three target hormones were readily extractable and measurable in a single HPLC analysis. Elution times for 15 separate fecal extracts averaged 16.59 ± 0.12 min for cortisol, 20.10 ± 0.09 min for corticosterone and 24.54 ± 0.10 min for testosterone. The nearest significant other peaks were >0.17 min from the average retention time of cortisol, corticosterone or testosterone. A representative HPLC elution profile of a fecal extract is presented in Fig. 3. In HPLC runs for eight aliquots of the same extract averaged for all three hormones, there was 5.20% variability in hormone concentration and 0.9% variability in retention time.

The validation procedures indicated that the eluent peaks presumed to contain a given steroid in fact did contain that steroid and that the measurement was quantitative (Table 1, Fig. 1 and Section 2).

Average levels for each hormone in 9 AM vs. 5 PM samples averaged across 7 days revealed similar levels between the 9 AM and 5 PM sample pools (Table 4). Also, assessment of hormone stability in fecal casts over 24 h revealed <10% change in any hormone level in pooled fecal sample incubated in 28 °C aquarium water for up to 16 h (Table 5).

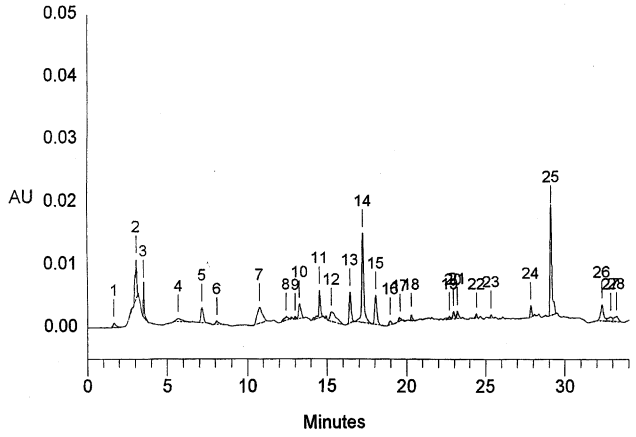

Fig. 3. A representative HPLC elution pattern for an extracted fecal cast from *S. guacamaia*. AU is absorbance units at 240 nm. Cortisol, peak 13; corticosterone, peak 18; and testosterone, peak 22.

Hormone values for contemporary plasma and fecal samples are presented in Table 6. Levels of corticosterone were greater than levels of cortisol and testosterone in both plasma and feces. Plasma and fecal levels of hormones were in different units of measure and not directly comparable. The plasma:fecal ratio was 2.9 for corticosterone, 1.8 for cortisol and 0.8 for testosterone.

3.1.2. Stress study

Levels of cortisol, corticosterone and testosterone averaged 3.4 ± 1.1, 14.8 ± 2.8, and 8.3 ± 1.2 ng/g feces, respectively, across the 32-day baseline period, with no trend observable (Fig. 4). During the hypoxia/restraint stress period, each stress event was associated with a marked elevation in cortisol and corticosterone, but not in testosterone (Fig. 4). In the interval between stress protocols, the cortisol and corticosterone levels returned to or near baseline levels. The average levels of cortisol, corticosterone and testosterone across the entire 13-day stress period were 36.9 ± 7.3, 48.2 ± 7.7, and 10.3 ± 1.8 ng/g feces. These average values for cortisol and corticosterone were 10.8-fold and 3.2-fold greater, respectively, than the cortisol and corticosterone average values in baseline. Average values in follow-up for cortisol (7.0 ± 1.5 ng/g feces) and corticosterone (25.1 ± 4.2 ng/g feces) were significantly (one-fifth and one-half, respectively) lower than those values in the stress period. Follow-up values averaged 2.0-fold (cortisol) and 1.7-fold (corticosterone) above baseline, and these differences were significant. In follow-up the average value for testosterone (11.5 ± 1.5 ng/g feces) was not different from testosterone values in the baseline and stress periods.

Table 4
Effect of time of day of fecal collection on steroid hormone levels in feces of aquarium-adapted *S. guacamaia*

Collection time	No. of days[a]	Hormone concentration (ng/g feces)[d]		
		Cortisol	Corticosterone	Testosterone
9 AM[b]	7	5.1 ± 1.7	14.0 ± 2.8	6.7 ± 1.9
5 PM[c]	7	7.2 ± 2.0	11.5 ± 2.6	3.9 ± 1.6

[a] Fecal samples from six fish were pooled to yield two pooled samples per day (9 AM and 5 PM). Samples were collected daily for 7 days.
[b] Samples contained all fecals produced from 5 PM to 9 AM.
[c] Samples contained all fecals produced from 9 AM to 5 PM.
[d] No differences between collection times for any hormone, Student's t test, $p > 0.05$.

Table 5
Effect of sample aging/incubation on steroid hormone levels in feces of aquarium-adapted *S. guacamaia*

Hours of sample aging/incubation[a]	Hormone concentration (% of fresh, zero-hour sample value)		
	Cortisol	Corticosterone	Testosterone
0	100	100	100
8	102	101	100
16	93	96	98
24	87	89	96

[a] Fresh samples ($n = 20$) were collected and immediately frozen, then simultaneously thawed, pooled and incubated in seawater at 28 °C. Aliquots were withdrawn at various times and assayed.

Table 6
Plasma and fecal levels of cortisol, corticosterone and testosterone in unstressed, aquarium-adapted *S. guacamaia*

Hormone type	No. of samples[a,b]	Hormone concentration	
		Plasma (ng/ml)	Feces (ng/g wet wt)
Cortisol	6	8.9 ± 3.1	3.1 ± 1.6
Corticosterone	6	18.6 ± 4.9	10.5 ± 3.8
Testosterone	6	5.6 ± 1.3	7.1 ± 2.2

[a] Fecal samples were taken from individual, isolated fish for 4 days prior to sedation/blood collection.
[b] Protocol repeated 10 days later and respective collection sets pooled per fish to insure sufficient plasma and sufficient fecal per fish for analysis.

3.2. Field study

Field values for Rainbow parrotfish averaged 2.0 ± 1.5, 8.9 ± 3.0, and 4.1 ± 0.9 for cortisol, corticosterone, and testosterone, respectively. These values represented nine pooled samples in each of three collection vials, with each vial providing a single value for each hormone. These values were not different from values for corresponding hormones in samples from rainbow parrotfish in the aquarium. Values for hormones in rainbow fish in the field were also not different from corresponding hormone values for pooled stoplight and queen parrotfish samples. Average hormone levels in extracts from vials containing queen parrotfish only or stoplight parrotfish only were not different for any hormone measured (Table 3).

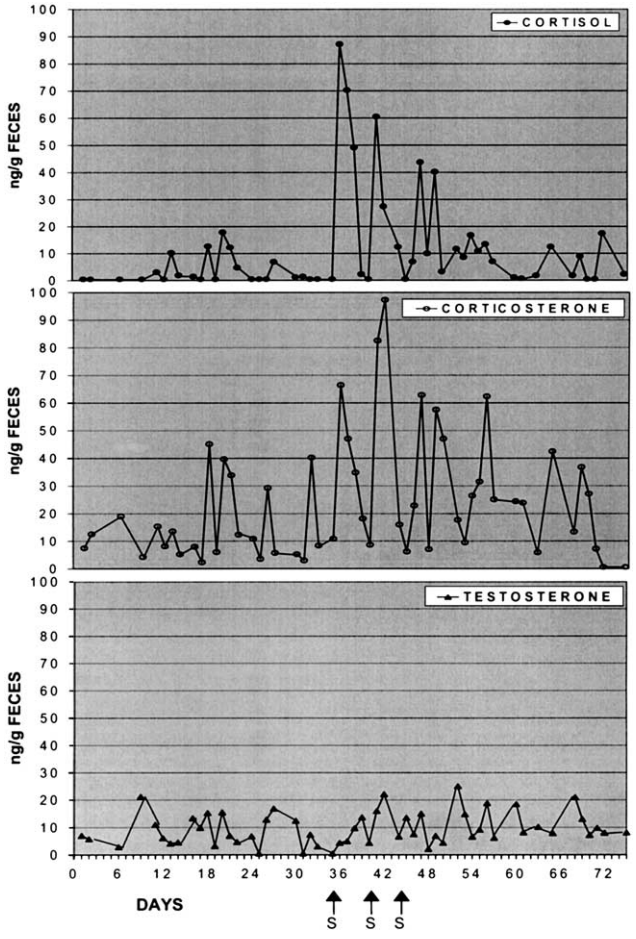

Fig. 4. Effects of acute, repetitive restraint/hypoxia stress on steroid hormone levels in fecal casts of aquarium-adapted *S. guacamaia*. Each point represents fecal casts pooled from six fish. Stress consisted of repeated netting and removal of fish from water over a 2-day period. All fish were exposed to this 2-day stress (*S*) protocol three times, as shown. Averages for cortisol and corticosterone (but not testosterone) were significantly ($p < 0.01$, paired-sample t test) greater in the stress period than in baseline or follow-up and were also greater ($p < 0.05$) in follow-up than in baseline.

Average values for fecal cortisol, corticosterone and testosterone at coral reef fecal-collection sites are presented in Fig. 5. The average values for extracts from vials containing pooled queen and stoplight parrotfish across all sites for cortisol, corticosterone and testosterone were respectively, 5.5 ± 1.8, 11.0 ± 3.1, and

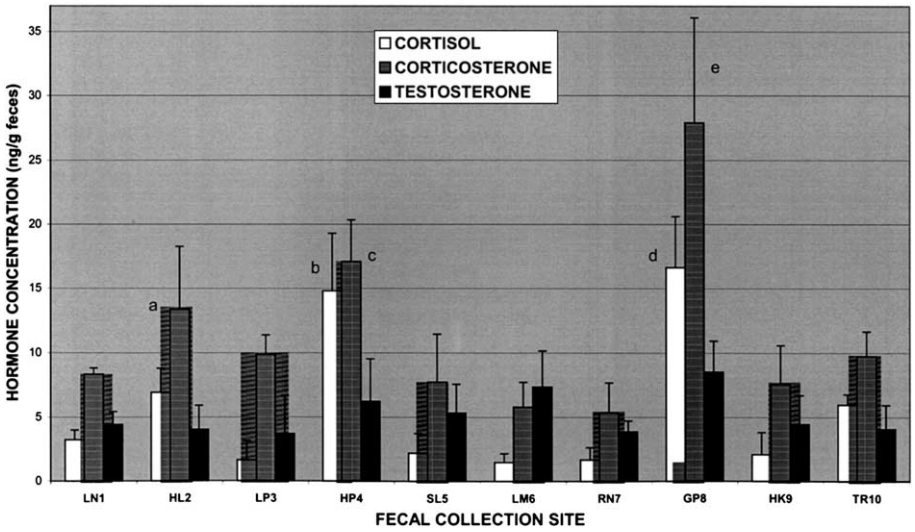

Fig. 5. Fecal steroid hormone levels in adult parrotfish (*S. viride* and *S. vetula*) inhabiting fringing reef of various bays around the Caribbean Island of St. John, US Virgin Island. Site names are abbreviated from map names and numbered as in Fig. 3. Each value is the average of one 6-fecal collection made on each of 3 days. Each consisted of six pooled fecal casts (three male, three female). The total collection period across all bays was three weeks (January/February 1999). For cortisol, b and d values were not different, but both were significantly ($p < 0.05$, Tukey test) greater than all others. For corticosterone, c and e were significantly greater than all others, except a and c were not different.

5.3 ± 2.1 ng/g. In two sites (Gallows Point and Harbor Point) the average for cortisol and for corticosterone was significantly greater than the respective cortisol or corticosterone average of any other sites. ANOVA revealed no differences in testosterone across sites.

Regarding site characteristics (Table 2), analysis by the Kruskal–Wallis test revealed no differences among sites for any characteristic. There was no significant correlation between any site characteristic and any hormone. Few correlations approached significance, although cortisol vs. turbidity was correlated at $p = 0.054$.

4. Discussion

4.1. Methodological

Organic extraction and HPLC analysis of parrotfish fecal casts was a reasonable means of determining their cortisol, corticosterone, and testosterone content. An advantage of this methodology over more recently validated RIA methods for fecal steroid hormone analysis (Wasser et al., 2000) is the ability to measure several hormones simultaneously in a single sample. However, HPLC is less efficient and generally less sensitive than RIA. The requirement for sample pooling to compensate for this lower sensitivity is a notable disadvantage of the HPLC method. Nonetheless, RIA was not a reliable method for measuring these hormones in feces when this work was done, and we used HPLC. It is possible that current immunoassays for mammalian fecal glucocorticoids (Wasser et al., 2000) can be adapted for individual-sample measurement of fish fecal glucocorticoids in the future.

In the present study we did not assess the metabolites of cortisol, corticosterone and testosterone which may have been present in parrotfish feces. In mammals the majority of glucocorticoids in feces are metabolites of the primary glucocorticoids released into the blood rather than the primary glucocorticoids themselves (Bahr et al., 2000; Vylitová et al., 1998). While the primary glucocorticoids such as cortisol and corticosterone may comprise a small percentage of glucocorticoids present in the parrotfish feces, they nonetheless were detectable and should serve as a reasonable measure of stress responding, regardless of the possible effects of other factors on glucocorticoid metabolism.

In the aquarium study, baseline variability was considerably less than the response to the stressor, which resulted in cortisol and corticosterone average levels which were respectively 10.8-fold and 3.2-fold greater than baseline average levels. Thus, it appears feasible to distinguish relatively acute stressful conditions from unstressed conditions via fecal hormone analysis, i.e., variability is not likely to mask acute response to stress. Whether differences would be detectable between basal fecal hormone levels and levels associated with mild chronic stress is unknown.

4.2. Aquarium

The marked responses of both fecal cortisol and corticosterone to stressors and the absence of this response by fecal testosterone suggest that the fecal steroid response is not a generalized hormone response to stress,

but rather a response specific to the interrenal axis. In the response to the stress protocol, cortisol evidenced greater lability than corticosterone. This responsiveness combined with lower baseline variability for cortisol (Fig. 4) suggests that cortisol may be more suitable for fecal monitoring of stress in parrotfishes.

Interestingly, both cortisol and corticosterone average levels in follow-up were significantly above baseline, despite the rapid return to near pre-stress levels within a day or 2 after each stress event. It is possible that the repetitive stresses altered glucocorticoid regulation, and that recovery from the endocrine disruption of repeated restraint/hypoxia stress was not complete until near the end of the 23-day follow-up when levels were again at baseline. Testosterone did not increase during the acute stress periods in the aquarium study, and the follow-up data in Fig. 4 suggest further that the testosterone levels were not influenced by sequela of repetitive stresses.

It is not known whether the hormone response patterns would differ across types of stressor, or whether the hypoxia/handling stress produced maximal response. The numerically lesser response in the third stress period (especially for cortisol, which showed $<50\%$ of the original response) raises the possibility that some adaptation to the response occurred.

Since neither fecal nor blood levels of the hormones of this study had been previously reported for parrotfish, we determined basal plasma hormone levels to rule out the possibility that fecal and blood hormone levels were grossly incongruous and to compare our plasma hormone findings with those in the fish literature for other species. Regarding plasma hormones, the sedation/blood sampling procedure was carried out in less than 5 min, which is less than the times reported for a detectable rise in plasma cortisol following stress in other fish species (Biron and Benfey, 1994; Grutter and Pankhurst, 2000; McCormick et al., 1998). It is thus likely that the plasma hormone levels represented baseline or minimal stress condition.

Average unstressed levels of plasma cortisol, corticosterone, and testosterone each showed a similar relationship to the fecal counterpart averages among individuals. This suggests that fecal measures of these hormones reflected plasma measures in the unstressed condition. Although corticosterone has received little attention in studies of teleost fishes, there are numerous reports on plasma levels of cortisol. The resting plasma cortisol levels in parrotfishes were reasonably consistent with plasma levels of this hormone reported for some other teleost species, including salmonids (Morrison et al., 1985; Sumpter et al., 1986), trout (Pottinger and Moran, 1993) and wrasse (Grutter and Pankhurst, 2000). The latter study also reported up to 45-fold greater levels of plasma cortisol in acutely stressed (capture, handling, and transport) as compared to unstressed wrasse (*Hemigymnos malapterus*). While the present study has focused on fecal glucocorticords, including their response to stress, further studies will be needed to address the plasma/fecal hormone relationship in response to stress. Such studies would strengthen the consideration of fecal glucocorticoids as an index of stress.

Several-fold higher resting levels of plasma cortisol have been observed for fish in captivity vs. free, field conditions (Grutter and Pankhurst, 2000; Pankhurst, 1998). Comparison of field vs. aquarium hormone values in rainbow parrotfish in the present study revealed no significant differences for any of the three measured hormones. Although numerous uncontrolled variables make this comparison tenuous, these results do not rule out the use of an aquarium environment for stress studies using stressors which may be relevant to the field.

The fact that fecal hormones did not degrade significantly during 16 h in the sample at room temperature is advantageous for field work, eliminating the need for ice in collection and transport. An advantage of remote fecal collection is that no stressful disturbance occurs to the individual fish or to the social structure of groups of fish, since no fish are handled or permanently removed from the site. This may be a significant consideration, since the social impact of individuals in a fish population is not well understood (Helfman and Schultz, 1984; Kramer et al., 1997) and alterations in social hierarchy may be stressful.

4.3. Field

In the field, six fish were sampled per data point, which may have contributed to lower variability than might be seen in individually monitored fish. Equal numbers of adult males and female samples were collected in each vial in order to minimize influence of possible sex differences in testosterone production and stress–response characteristics. The initial purpose of pooling fecal samples was to insure sufficient fecal material for hormone assay. All samples on a given day were from different individual fish. It is not known to what extent collections from day to day were from the same fish. However, the observed territoriality of some parrotfish and the small size of collection sites in this study make this a possibility.

The results of the comparison of queen and stoplight parrotfish data (Table 3) validated the pooling of samples between these species, and collection of equal numbers of male and female samples mitigated the issue of possible sex differences. One potential disadvantage of pooling is the possibility that one or two stressed fish in a given pooled sample could elevate the value for that sample. We attempted to address this issue in the field by collecting three pooled samples across 3 weeks at each site. We assumed that the probability of such a skewing effect would be similar across sites and samples. Thus, the average value for each site in Fig. 5 is from an n of 3, which is not a pooled n. Likewise, in the lab study several sets of multiple samples from the same fish were

obtained to permit comparison of fecal and plasma levels of the target hormones.

The purpose of sampling several field sites was to obtain basic data on hormone-level averages and their variability from site to site. The finding of markedly elevated cortisol and corticosterone in parrotfish fecals in 2 of the 10 sampled bays was unexpected. The absence of differences in any site characteristics across sites and the lack of hormone/site-characteristic correlations precluded any perspective regarding the basis for these hormone differences. However, the n was small, intended primarily to examine the possibility of gross site differences, and it is possible that existing differences were masked by variability.

It is noteworthy in comparing Figs. 2 and 5 that the two sites exhibiting significantly greater cortisol and corticosterone levels than other sites were those sites associated with greatest human development. In our opinion this finding warrants further study. Another important aspect of finding hormone-level differences among bays is that the background variability in these hormone levels was not so great as to mask observation of differences in hormone levels among field sites.

Stress in individual fish can compromise aspects of their immune function (Maule et al., 1989; Narnaware et al., 1994; Pickering, 1993), growth (McCormick et al., 1998; Pickering, 1993) and reproduction (Campbell et al., 1992; Morrison et al., 1985). Thus, entire fish populations inhabiting a given site and exposed to repetitive or chronic stressors could be subject to compromise. The results of the present study encourage further examination of the hypothesis that fecal steroid hormone measurement can be used for remote stress assessment of parrotfishes in the field.

Acknowledgments

The authors express appreciation to the following organizations and persons: Virgin Islands National Park for collection permits and logistical support (S. Caseau, J. Hopkins, T. Kelley, and R. Waara), University of the Virgin Islands and Virgin Island Environmental Resource Station (V. Powell and R. Brown) for provision of air compressor, air tanks, and logistical/technical support; Toledo Zoo Aquarium (J. Hemdal) for aquarium advisory support and technical assistance; A. Anthony, R. Bremser, A. Karsten, T. Kelley, R. Myers, V. Powell, and J. Roby for field assistance and C. Kern-Buell for laboratory assistance.

References

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

Bahr, N.I., Palme, R., Mohle, U., Hodges, J.K., Heistermann, M., 2000. Comparative aspects of the metabolism and excretion of cortisol in three individual nonhuman primates. Gen. Comp. Endocrinol. 117, 427–438.

Barton, B.A., Bollig, H., Hauskins, B.L., Jansen, C.R., 2000. Juvenile pallid (*Scaphirhynchus albus*) and hybrid pallid × shovelnose (*S. albus* × *platorynchus*) sturgeons exhibit low physiological responses to acute handling and severe confinement. Comp. Biochem. Physiol. 126, 125–134.

Barton, B.A., Iwama, G.K., 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.

Biron, M., Benfey, T.J., 1994. Cortisol, glucose and hematocrit changes during acute stress, cohort sampling, and the diel cycle in diploid and triploid brook trout (*Salvelinus fontinalis Mitchill*). Fish Physiol. Biochem. 13, 153–160.

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

Bythell, J.C., Gladfelter, E.H., Bythell, M., 1993. Chronic and catastrophic natural mortality of three common Caribbean reef corals. Coral Reefs 12, 143–152.

Campbell, P.M., Pottinger, T.G., Sumpter, J.P., 1992. Stress reduces the quality of gametes produced by rainbow trout. Biol. Reprod. 47, 1140–1150.

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

Grutter, A.S., Pankhurst, N.W., 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.

Helfman, G.S., Schultz, E.T., 1984. Social transmission of behavioral traditions in coral reef fish. Anim. Behav. 32, 379–383.

Hixon, M.A., Beets, J., 1993. Predation, prey refuges, and the structure of coral-reef fish assemblages. Ecol. Monogr. 63, 77–101.

Hughes, T.P., 1994. Catastrophes, phase shifts, and large-scale degradation of a Caribbean coral reef. Science 265, 1547–1551.

Hunte, W., 1992. Effects of eutrophication and sedimentation on juvenile corals. II. Settlement. Mar. Biol. 114, 625–631.

Iwama, G.K., Pickering, A.D., Schreck, C.B., 1997. Stress and Health in Aquaculture. Cambridge University Press, UK.

Jurke, M.H., Czekala, N.M., Lindburg, D.G., Millard, S.E., 1997. Fecal corticoid metabolite measurement in the cheetah (*Acinonyx jubatus*). Zoo Biol. 16, 113–147.

Kirkpatrick, J.F., Liu, I.K.M., Turner Jr., J.W., 1990. Remotely-delivered immunocontraception in feral horses. Wildlife Soc. Bull. 18, 326–330.

Kramer, D.L., Rangeley, R.W., Chapman, L.J., 1997. Habitat selection: patterns of spatial distribution from behavioral decisions. In: Godin, J.-G.J. (Ed.), Behavioral Ecology of Teleost Fishes. Oxford University Press, Oxford, pp. 37–80.

Lasley, B.L., Kirkpatrick, J.F., 1991. Monitoring ovarian function in captive and free-ranging wildlife by means of urinary fecal steroids. J. Zoo. Wildlife Med. 22, 23–31.

Maule, A.G., Tripp, R.A., Kaattari, S.L., Schreck, C.B., 1989. Stress alters immune function and disease resistance in Chinook salmon (*Oncorhynchus tshawytsha*). J. Endocrinol. 120, 135–142.

McCormick, S.D., Shrimpton, J.M., Carey, J.B., O'Dea, M.F., Sloan, K.E., Moriyama, S., Bjornsson, B.T., 1998. Repeated acute stress reduces growth rate of Atlantic salmon parr and alters plasma levels of growth hormone, insulin-like growth factor I and cortisol. Aquaculture 168, 221–235.

Miller, M.W., Hobbs, N.T., Sousa, M.C., 1991. Detecting stress responses in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*); reliability of cortisol concentrations in urine and feces. Can. J. Zool. 69, 15–24.

Morrison, P.F., Leatherland, J.F., Sonstegard, R.A., 1985. Plasma cortisol and sex steroid levels in Great Lakes coho salmon

(*Oncorhynchus kisutch* Walbaum) in relation to fecundity and egg survival. Comp. Biochem. Physiol. 80, 61–68.

Mulrow, P.J., 1986. The Adrenal Gland. Elsevier, New York.

Narnaware, Y.K., Baker, D.L., Tomlison, M.G., 1994. The effect of various stresses, corticosteroids and adrenergic agents on phagocytosis in the rainbow trout *Oncorhynchus mykiss*. Fish Physiol. Biochem. 13, 31–40.

Pankhurst, N.W., 1998. Reproduction. In: Black, K.D., Pickering, A.D. (Eds.), Biology of Farmed Fish. Sheffield Academic Press, Sheffield, pp. 1–26.

Pickering, A.D., 1993. Growth and stress in fish production. Aquaculture 111, 51–63.

Pottinger, T.G., Moran, T.A., 1993. Differences in plasma cortisol and cortisone dynamics during stress in two strains of rainbow trout (*Oncorhynchus mykiss*). J. Fish Biol. 43, 121–130.

Redding, J.M., 1993. Influence of ambient salinity on osmoregulation and cortisol concentration in yearling Coho salmon during stress. Trans. Am. Fisheries Soc. 112, 800–807.

Richmond, R.H., 1993. Coral reefs: present problems and future concerns resulting from anthropogenic disturbances. Am. Zool. 33, 524–536.

Rogers, C.S., 1990. Responses of coral reefs and reef organisms to sedimentation. Mar. Ecol. Prog. Ser. 62, 185–202.

Rogers, C.S., Beets, J., 2001. Degradation of marine ecosystems and decline of fishery resources in marine protected areas in the U.S. Virgin Islands. Environ. Conserv. 28, 312–322.

Rogers, C.S., Garrison, G., Grober, R., Hills, Z., Franke, M., 1994. Coral reef monitoring manual for the Caribbean and Western Atlantic. National Park Service Publication, Atlanta.

Russ, G.R., 1991. Coral reef fisheries: effects and yields. In: Sale, P.F. (Ed.), The Ecology of Fishes on Coral Reefs. Academic Press, San Diego, pp. 601–635.

Safer-Hermann, N., Ismail, M.N., Choi, H.S., Mostl, E., Bamberg, E., 1987. Pregnancy diagnosis in zoo animals by estrogen determination in feces. Zoo Biol. 6, 189–193.

Schreck, C.B., 1990. Biological Indicators of Stress in Fish. American Fisheries Society, Bethesda.

Stoskopf, M.K., 1993. Fish Medicine. W.B. Saunders, Philadelphia, London.

Sumpter, J.P., Dye, H.M., Benfey, T.J., 1986. The effects of stress on plasma ACTH, alpha-MSH, and cortisol levels in salmonid fishes. Gen. Comp. Endocrinol. 62, 377–385.

Sunyer, J.O., Gomez, E., Navarro, V., Quesada, J., Tort, L., 1995. Physiological responses and depression of humoral components of the immune system in gilthead sea bream (*Sparus aurata*) following daily acute stress. Can. J. Fish Aquat. Sci. 52, 2339–2346.

Turner Jr., J.W., 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 Wildlife Med. 33, 214–221.

Vylitová, M., Miksik, I., Pácha, J., 1998. Metabolism of corticosterone in mammalian and avian intestine. Gen. Comp. Endocrinol. 109, 315–324.

Wasser, S.K., Hunt, K.E., Brown, J.L., Cooper, K., Crockett, C.M., Bechert, U., Millspaugh, J.J., Larson, S., Monfort, S.L., 2000. A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. Gen. Comp. Endocrinol. 120, 260–275.

Whitten, P.L., 1997. Noninvasive methods for the study of stress, reproductive function, and aggression. Am. J. Physiol. Anthropol. 24, 239.

Wilkinson, C., 2002. Status of Coral Reefs of the World: 2000. Australian Institute of Marine Science, Queensland.

Wolff, N., Grober-Dunsmore, R., Rogers, C.S., Beets, J., 1999. Management implications of fish trap effectiveness in adjacent coral reef and gorgonian habitats. Environ. Biol. Fishes 55, 81–90.

Zar, J.H., 1984. Biostatistical Analysis, second ed. Prentice-Hall, Englewood Cliffs, NJ.