

Measuring water-borne cortisol in convict cichlids (*Amatitlania nigrofasciata*): is the procedure a stressor?

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(Accepted: 8 July 2008)

Summary

Cortisol is an important indicator of health and behavioral state in fishes, and is produced in response to stressors including confinement, handling and social conflict. An inherent difficulty in measuring circulating cortisol is the implementation of invasive procedures that can be potent stressors. Recent studies show that cortisol can be reliably quantified from fish holding water by placing individuals in a small beaker for a predetermined collection period. We investigated whether convict cichlid fish (*Amatitlania nigrofasciata*) mount a significant stress response to beaker confinement and whether they habituate to the collection method. We also determined the relationship between plasma and water-borne cortisol, and changes in cortisol release rates following handling and cortisol administration. Initial beaker exposure induced high cortisol release rates but cichlids quickly habituated after 3–4 exposures. We revealed significant positive correlations between plasma and water-borne cortisol, and marked increases in water-borne cortisol release rates after cortisol injection that persisted for between 4 and 24 h, depending on the dosage. In conclusion, we provide convincing evidence for the utility and validity of the water-borne collection method to measure cortisol release rates in convict cichlids.

Keywords: cortisol, water-borne, plasma, confinement, stress, convict cichlid.

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Introduction

Collection of water-borne steroid hormones has become an increasingly popular method for measuring concentrations of androgens, estrogens, glucocorticoids, and many pheromonally active steroidal compounds in fishes (Scott & Ellis, 2007). This technique is a relatively non-invasive alternative to bleeding, which is especially important for research concerned with evaluating stress hormone (e.g., cortisol) concentrations and/or temporal changes in steroid hormone concentrations; in these cases multiple sessions of bleeding might induce a stress response and/or be detrimental to the experimental animals. The first studies to examine water-borne cortisol levels (Ruane & Komen, 2003; Ellis et al., 2004) pumped water from large enclosures containing groups of rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). Both demonstrated that water-borne cortisol increased in a predictable manner following exposure to handling (Ellis et al., 2004) or stocking (Ruane & Komen, 2003) stressors. Ellis et al. (2004) also showed significant concordance between plasma and water-borne cortisol concentrations in the rainbow trout. Although sampling from group tanks can be insightful for many questions related to aquaculture and welfare practices in fishes, it does not allow one to explore the causes for, or implications of individual variation in endocrine status.

The water-borne hormone collection technique recently has been adapted to investigate individual steroid release rates (e.g., pg/g per h) in response to various behavioral and environmental stimuli in small fishes. For instance, the procedure has been applied to explore covariance between a number of steroid hormones (e.g., testosterone, 11-ketotestosterone (KT), androstenedione (Ad), 17 β -estradiol (E2), cortisol) and reproductive or aggressive behavior in stickleback (*Gasterosteus aculeatus*, Sebire et al., 2007), blue-banded gobies (*Lythrypnus dalli*, Black et al., 2005; Rodgers et al., 2006; Lorenzi et al., 2008), Siamese fighting fish (*Betta splendens*, Dziejewczynski et al., 2006), mangrove killifish (*Kryptolebias marmoratus*; Earley & Hsu, 2008), and both African and South American cichlids (Hirschenhauser et al., 2004; Bender et al., 2006; Earley et al., 2006). In addition, Wysocki et al. (2006) found that ship noise elicits significantly elevated water-borne cortisol concentrations in individual European perch (*Perca fluviatilis*), common carp (*Cyprinus carpio*), and gudgeons (*Gobio gobio*). Lower et al. (2005) also demonstrated a marked, rapid increase in water-borne cortisol following the

implantation of transmitters in individuals of both common carp and roach (*Rutilus rutilus*). These new individual-based applications of the water-borne collection technique are of great significance because they allow for hormone sampling in small species that would otherwise have to be terminally bled or fast-frozen for whole body assays. For larger species, the technique provides a less invasive means of obtaining steroid hormone concentrations. This method, thus, opens a new niche for research in behavioral and applied endocrinology, especially in small fishes — namely the ability to quantify relationships between hormones and behavioral (e.g., confrontation), environmental (e.g., noise) or anthropogenic (e.g., tagging) stimuli over a short time course (e.g., hours or days) with repeated sampling. Indeed, Sebire et al. (2007) successfully evaluated KT, Ad, and cortisol concentrations in stickleback both before and after mating attempts (45 min interval) by transferring the fish for 30 min to a small beaker.

Despite the attractiveness of this method for use in small fishes, there are some potential limitations that remain unaddressed, especially for measuring stress hormones. In particular, the procedure involves transferring fish from a relatively large home tank or experimental aquarium to a small beaker (or jar) for hormone collection. Confinement, however, is known to induce significant acute stress responses in fish (e.g., Pottinger et al., 1992; Pottinger & Carrick, 2001; Barreto et al., 2006). Therefore, if the aim of collecting water-borne hormones is to determine baseline cortisol concentrations, it is imperative to understand whether (1) the beaker placement procedure triggers an acute stress response such that initial water-borne collections reflect stress responsive rather than baseline cortisol concentrations (see Earley et al., 2006) and (2) fishes habituate to this potential confinement stressor. The primary objective of this study was to generate a habituation protocol that would allow for measurement of both stress responsive and baseline cortisol concentrations in the convict cichlid fish (*Amatitlania nigrofasciata*). The second goal of our study was to determine whether cortisol release rates into the water reflect circulating plasma cortisol concentrations. Third, we evaluated whether exogenous manipulation of the stress axis with cortisol would be visible as increased cortisol concentrations in water extracts.

Convict cichlids were chosen as a model because they exhibit a rich behavioral repertoire, including intense territorial aggression, pairbond formation, biparental care of offspring, and brood adoption (e.g., Koops & Grant, 1993; Mackereth & Keenleyside, 1993; Wisenden, 1995; Draud & Lynch,

2002; Itzkowitz et al., 2005; Santangelo, 2005; van Breukelen & Draud, 2005). Only one study, however, has examined the possible endocrine correlates of these behaviors (Earley et al., 2006). Although convict cichlids, especially males, can grow to be quite large (30–35 g), they are reproductively mature and display all of the aforementioned behaviors at much smaller sizes (3–10 g), where repeated bleeding is not practical. The water-borne hormone collection method has the potential to significantly advance our knowledge of the hormonal basis of behavior in a species — the convict cichlid — that has been established as a model system in ethology and behavioral ecology.

Methods

Study organism and maintenance

Adult convict cichlids were obtained from Pet Solutions (Beavercreek, OH, USA) and housed in the laboratory as mixed sex groups of 30–35 individuals in two 284-l holding aquariums maintained at 24°C ($\pm 2^\circ\text{C}$) with chemical and biological filtration, a gravel substrate, and over-abundant shelter (broken terra cotta pots); a total of $N = 110$ adult male/female cichlids were used for the three experiments described herein. Photoperiod was maintained at 12:12 h light/dark using fluorescent and incandescent lights. Fish were fed once daily between 1000 and 1100 h, or after experimental procedures, with a mixture of frozen bloodworms, frozen brine shrimp and cichlid flakes. The Institutional Animal Care and Use Committee at California State University Fresno (File No. 127) approved all procedures described herein. Individual convict cichlids were used only once and were transferred to new 284-l holding aquariums (different than original housing) after experimentation to eliminate repeated sampling.

Experiment 1: habituation to beaker confinement

We conducted two separate rounds of the habituation procedure. In the first round, adult female ($N = 11$) convict cichlids were subjected to three successive days of beaker confinement. In the second round, adult males ($N = 19$) were subjected to four successive days of beaker confinement. We used a fourth day of beaker confinement to determine whether habituation would extend past three days; only males were used in the second round due to limited female population size in the laboratory.

In both rounds, individuals were transferred from the holding aquariums to separate 37-l tanks with the same basic conditions as the holding aquariums, except with only one terra cotta shelter. After 4 days acclimation to individual tanks, each fish was transferred, by netting, to a clean 600 ml beaker filled with 300 ml of clean water for 30 min to collect water-borne hormones; 30 min collection times have recently been used with success in stickleback (Sebire et al., 2007). All hormone collection and transfer (see below) beakers were cleaned with 100% ethanol and distilled water. The initial transfer occurred between 1000 and 1015 h and the handling time (i.e., from first insertion of the net into the tank until the fish was placed into the beaker) was recorded for all fish. After 30 min confinement (hormone collection), the contents of the hormone collection beaker (including the fish), were poured through a net (washed in distilled water) into a new, clean 600 ml transfer beaker. The fish, which were captured in the net, were returned to their respective tanks and treated with Stress Coat™ to replenish slime coating. This procedure was repeated on three (Round 1) or four consecutive days (Round 2), at the exact same time of day. After the last (third or fourth) day of hormone collection, the fish were weighed, measured using vernier calipers, and sex (male or female) was verified by examination of genital papilla morphology (male papillae are pointed, while female papillae are ovate) (Table 1). Standard length was measured from the tip of the snout to the caudal peduncle, total length from the tip of the snout to the posterior edge of the caudal fin, and body depth from the anterior origin of the dorsal fin to the genital papilla. Water samples were processed immediately after collection using methods described in detail below (see also Table 2).

Experiment 2: handling stress and plasma versus water correlations

We conducted two separate rounds of this experiment to evaluate whether being held out of the water in a net would increase water-borne and plasma cortisol relative to control animals. In Round 1, female cichlids were transferred from the holding aquariums to individual 37-l tanks equipped with one terra cotta shelter for 4 days, and individuals were randomly assigned to the 'no handling' ($N = 7$) or 'handling' ($N = 9$) treatment. On day 5, animals were transferred either directly by netting from their individual tank to a clean 600 ml beaker filled with 300 ml clean water (no handling), or were captured from their individual tank, held out of water immobilized in

Table 1. Summary of body measurements for animals used in Experiments 1–3.

Experiment	Sex	<i>N</i>	Mass (g)	Total length (mm)	Standard length (mm)	Body depth (mm)
Experiment 1						
Round 1	♀	11	3.26 ± 0.31	55.36 ± 1.98	42.95 ± 1.65	18.01 ± 0.71
Round 2	♂	19	19.64 ± 1.22	99.64 ± 1.88	79.73 ± 1.57	33.39 ± 0.76
Experiment 2						
Round 1						
Not handled	♀	7	5.05 ± 0.31	64.19 ± 1.49	51.44 ± 1.26	24.89 ± 0.51
Handled	♀	9	4.02 ± 0.23	59.41 ± 1.35	46.51 ± 1.11	19.37 ± 0.37
Round 2						
Not handled	♂	13	17.72 ± 2.04	92.02 ± 3.47	79.98 ± 4.58	32.21 ± 4.03
Handled	♂	12	18.56 ± 1.57	87.15 ± 7.93	83.47 ± 3.95	32.23 ± 1.08
Experiment 3						
CNTRL	♂	5	5.24 ± 0.44	63.80 ± 2.34	49.84 ± 1.68	22.40 ± 0.63
	♀	4	3.04 ± 0.11	54.02 ± 0.68	42.26 ± 0.45	17.96 ± 0.73
SHAM	♂	5	6.21 ± 1.15	68.08 ± 4.10	52.68 ± 3.53	24.00 ± 1.24
	♀	4	4.61 ± 0.27	62.00 ± 1.73	48.14 ± 1.33	21.94 ± 0.57
CORTLO	♂	6	5.45 ± 0.65	65.60 ± 2.61	50.43 ± 1.88	23.32 ± 0.81
	♀	4	5.12 ± 0.36	64.05 ± 1.68	49.80 ± 1.53	22.68 ± 0.53
CORTHI	♂	6	6.30 ± 0.58	70.30 ± 2.71	54.53 ± 2.43	24.07 ± 0.46
	♀	5	3.98 ± 0.42	57.37 ± 1.55	44.58 ± 1.07	20.95 ± 0.67

All values are represented as mean ± SEM.

the net for 2 min, and then placed in the 600 ml beaker (handling). Hormone collection began between 1000 and 1015 h for all animals and continued for 30 min.

In the second round, only male cichlids were used ($N = 13$ no handling, $N = 12$ handling) due to limited female population size in the laboratory. The procedure was the same as described for Round 1 except that fish were held out of water immobilized in a net for 3 min. After the 30 min hormone collection period, males were immediately exposed to an anesthetic dose of sodium bicarbonate buffered tricaine methane sulfonate (MS-222; 1 g/l), and blood was obtained by caudal venipuncture (27G heparinized syringe). Blood was maintained on ice until centrifugation at 17×10^3 rpm for 5 min; plasma was collected and processed using methods detailed below (Table 2). Plasma was not obtained from $N = 4$ males in Round 2, and the degrees of

Table 2. Summary of extraction methods used for Experiments 1–3 on both water-borne and plasma samples.

Experiment	Type	Solid phase extraction column	Elution solvent	Further processing	Reconstitution	
Experiment 1	Round 1	Water-borne	LiChrolut® RP-18 (500 mg, 4.0 ml)	Methanol	Evaporated 37°C	EIA buffer, 800 µl
	Round 2	Water-borne	LiChrolut® RP-18 (500 mg, 4.0 ml)	Methanol	Evaporated 37°C and ether extracted	EIA buffer, 800 µl
Experiment 2	Round 1	Water-borne	LiChrolut® RP-18 (500 mg, 4.0 ml)	Methanol	Evaporated 37°C and ether extracted	EIA buffer, 800 µl
	Round 2	Water-borne	LiChrolut® RP-18 (500 mg, 4.0 ml)	Methanol	Evaporated 37°C and ether extracted	EIA buffer, 800 µl
	Round 2	Plasma	None	None	Ether extracted	EIA buffer, 100× plasma volume
Experiment 3	Water-borne	Sep-Pak® Plus C18 (500mg, 4.0 ml)	Ethyl acetate + methanol	Evaporated 37°C	EIA buffer, 800 µl	
	Plasma	Sep-Pak® Plus C18 (500mg, 4.0 ml)	Ethyl acetate + methanol	Evaporated 37°C	EIA buffer, 100× plasma volume	

freedom in the statistical analyses reflect this. Water-borne hormones from both rounds were processed immediately after collection as described below (Table 2). All fish were measured (mm), weighed (g) and sexed (genitalia) before initial isolation (Table 1).

Experiment 3: administration of exogenous cortisol

Thirty-nine adult male (M) and female (F) convict cichlids were used to evaluate the effects of exogenous cortisol administration on water-borne cortisol concentrations. Animals were assigned to one of four treatments: (1) unmanipulated control (CNTRL; 5M, 4F), (2) sham (SHAM; 5M, 4F), (3) cortisol low dose (CORTLO; 6M, 4F), or (4) cortisol high dose (CORTHI; 6M, 5F). We prepared the injection solution by mixing a given mass of crystalline cortisol (hydrocortisone; Catalog H4001, Sigma-Aldrich, USA) with a vehicle, sesame oil. We employed two cortisol doses — low ($1 \mu\text{g}$ cortisol/g body mass) and high ($100 \mu\text{g}$ cortisol/g body mass). Previous studies have successfully used doses ranging from 0.3 to $110 \mu\text{g}$ cortisol/g body mass in fishes (Milligan, 2003; Tripathi & Verna, 2003; DiBattista et al., 2005); these studies utilized coconut oil, saline, and cocoa butter to produce their injection solution. Because sesame oil is a liquid at room temperature and readily dissolves steroids, we anticipated that this would promote, relative to other vehicles, our aim of inducing an acute spike of cortisol. The volume of injection was based on a 10 g fish receiving a $50 \mu\text{l}$ injection; smaller fish received less injection volume but the same concentration.

The initial procedure for Experiment 3 followed that of the beaker habituation experiment. Briefly, animals were transferred from the holding aquariums to individual 37-l tanks and allowed 4 days acclimation. Before treatment exposure, subjects were transferred by netting to clean 600 ml beakers filled with 300 ml clean water for 30 min on three successive days (initial transfer occurred between 1000 and 1015 h each day). Immediately following hormone collection on day 3, individuals were anesthetized in buffered MS-222 (1.5 g/l), and injected intraperitoneally (27G syringe) with low (CORTLO) or high (CORTHI) doses of cortisol, vehicle alone (sesame oil; SHAM), or nothing (replaced in their home tank unmanipulated following anesthesia; CNTRL). Water-borne hormones were then collected at 1, 4, 24 and 48 h post-treatment using the same methodology as described above; 24 and 48-h samples were obtained between 1000 and 1015 h. Water-borne

hormones were extracted immediately after all pre-treatment (days 1–3) and post-treatment (1, 4, 24, 48 h) time points. Immediately following the 48 h water-borne hormone collection, a subset of subjects were anesthetized and blood was drawn by caudal venipuncture using a 27G heparinized syringe. Blood was maintained on ice until centrifugation at 17×10^3 rpm for 5 min; plasma was collected and processed as detailed below.

Hormone extraction, processing and assay

Immediately after sample collection, the 300 ml water borne hormone samples were filtered with Whatman Filter paper 1, and passed through LiChrolut[®] RP-18 solid phase extraction columns (500 mg, 4.0 ml; Merck; Experiments 1 and 2) or Sep-Pak[®] Plus C18 columns (500 mg, 4.0 ml; Waters, USA; Experiment 3) (Table 2). Earley et al. (2006) describe the extraction protocol in detail. Briefly, columns were primed with 2×2 ml HPLC-grade methanol (MeOH) followed by 2×2 ml distilled water; water samples were passed through Tygon[®] tubing (Saint-Gobain, formulation 2275, ID = 1/16, OD = 3/16, Wall = 1/16) into the columns using a vacuum manifold, and once the entire water sample was extracted, the columns were washed with 2×2 ml distilled water.

Table 2 describes the different elution and extraction methods performed in each experiment. In summary, hormone was eluted from the column into 12×75 mm borosilicate vials with methanol (2×2 ml) to obtain total (free + conjugated) cortisol (Experiments 1 and 2) or into two, 12×75 mm borosilicate vials with successive, separate ethyl acetate (2×2 ml) and methanol (2×2 ml) washes to partition free and conjugated steroids, respectively (Experiment 3; Ellis et al., 2004). Eluted samples were stored at -20°C until further processing. Methanol or ethyl acetate was evaporated under a gentle stream (approx. 0.7 bar) of nitrogen in a water bath at 37°C , leaving a residue of hormone. Evaporated samples from Experiment 1 (Round 2) and Experiment 2 were resuspended in $200 \mu\text{l}$ of distilled water and free steroids were obtained by ether extraction (Table 2). Ether extraction entailed adding 2 ml diethyl ether (HPLC grade) to the residue reconstituted in distilled water, vortexing for 4 min, allowing phase separation for 2 min, fast freezing in a dry ice-MeOH bath, and decanting the ether layer into a 12×75 mm borosilicate vial. The remaining aqueous layer was thawed, subject to the procedure again, and the ether layer was decanted into the same borosilicate

vial as the first; ether was evaporated under nitrogen at 37°C in a water bath. All free hormone samples were resuspended in 800 μ l EIA buffer provided by a commercially available EIA kit purchased from Cayman Chemicals.

Plasma samples were processed either by ether extraction (see previous paragraph) on a given volume of plasma reconstituted in 100 μ l distilled water (Experiment 2, Round 2) or by dilution of plasma in 4 ml distilled water followed by the solid phase extraction procedure using Sep-Pak® Plus C18 columns and consecutive elution with ethyl acetate and methanol (Experiment 3) (Table 2). Extracted plasma samples were re-suspended in 100 \times original plasma volume; assayed concentrations were corrected for dilution factor in all cases. Manufacturer's procedures for the EIA were strictly followed.

A pooled convict cichlid water-borne hormone extract (approx. 45 ml generated from 100 non-experimental animals) was used to validate the EIA kit. A small portion (300 μ l) of this pool was serially diluted (1 : 1 through 1 : 128) and the slope of this dilution curve was parallel to the curve created with kit standards (compare slopes, Zar, 1996, p. 355: $t_{12} = -0.11$, $p = 0.99$). We also spiked 150 μ l of the cichlid pool with an equal volume of each standard to ascertain recovery; the slope of the curve plotting observed versus expected cortisol concentrations was 0.99, indicating a significant linear relationship. Minimum recovery was 93.4%. Twenty-two 96-well plates were assayed at three different times (assay 1, 10 plates; assay 2, 5 plates; assay 3, 7 plates); all samples were run in duplicate. Pooled extract from non-experimental animals was used as the intra- and inter-assay control; different aliquots of the pooled extract were used for the three assays. Intra-assay coefficients of variation were calculated from two, pooled cichlid extracts run in duplicate at the beginning and end of each EIA plate. These intra-assay coefficients of variation ranged from 0–31% (median = 5.2%, mean \pm SEM = 8.9 \pm 2.3%). Inter-assay coefficients of variation for the three assays were 10.56%, 7.34%, and 20.34%, respectively.

Statistical analyses

A mixed model repeated measures analysis of covariance (ANCOVA) evaluated changes in cortisol levels across habituation periods (Experiment 1) or before and after cortisol/sham manipulation (Experiment 3). Handling time (from home tank to collection beaker) was used as the covariate in all analyses. For Experiment 1, Rounds 1 and 2 were analyzed separately because

they employed different hormone extraction techniques and included only females or only males, respectively; in each analysis, day was the within subjects variable. For Experiment 3, treatment (e.g., CNTRL, CORTLO) and sex were entered as between subjects variables and time (7 pre- and post-injection periods) as the within subjects variable. Treatment or sex differences over time (i.e., interaction terms) were evaluated with least squares means contrasts, and the p values were subjected to sequential Dunn–Sidak adjustments to account for compounding of Type I error.

For Experiment 2, Rounds 1 and 2 of the handling stress experiment were analyzed separately because they were conducted at different times and on different sexes. Analyses of variance for treatment effects (no handling, handling) were conducted for both water-borne and plasma cortisol. Analysis of covariance assessed the relationship between water-borne (pg/g per h) and plasma (pg/ml) cortisol. Treatment was the independent variable, plasma cortisol was the covariate, and water-borne cortisol was the dependent variable; the non-significant treatment \times plasma cortisol ($F_{1,17} = 0.02$, $p = 0.89$) interaction was dropped to generate a reduced model.

Assumptions of ANCOVA (e.g., independence of covariate and main effects) were met. Covariance structures for the mixed model repeated measures ANCOVA were evaluated with Akaike's Information Criteria for best fit using a SAS macro. Cortisol concentrations were normally distributed without transform for Experiment 1 (Round 1) but were natural-log transformed to achieve normality in all other analyses. All analyses were conducted on SAS v9 and JMP v5.0.1 (SAS Institute, USA) with α set at 0.05.

Results

Experiment 1: Habituation to beaker confinement

The first round revealed that female convict cichlids habituate to beaker confinement over three successive exposures, as shown by a significant decrease in cortisol concentrations from day 1 to day 3 (day effect: $F_{2,29} = 17.52$, $p < 0.0001$; Figure 1); there were no significant effects of handling time ($F_{1,29} = 2.92$, $p = 0.10$). The results for males in Round 2 mirrored those from females in Round 1, with a significant decrease in confinement-induced cortisol concentrations from day 2 to day 4 ($F_{3,70} = 6.28$, $p = 0.0008$; Figure 2), and no effect of handling ($F_{1,70} = 0.5$, $p = 0.48$).

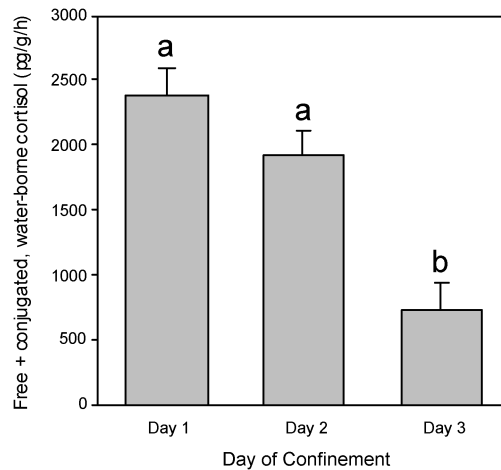


Figure 1. Change in total (free + conjugated) water-borne cortisol release rates across three successive days of beaker confinement in female convict cichlids (Round 1). Histogram bars (mean \pm SEM) with different letter notations are significantly different after sequential Dunn–Sidak adjustment of least squares mean contrasts (day 1 vs. day 2: $p = 0.28$; day 1 vs. day 3: $p < 0.0001$; day 2 vs. day 3: $p = 0.0005$).

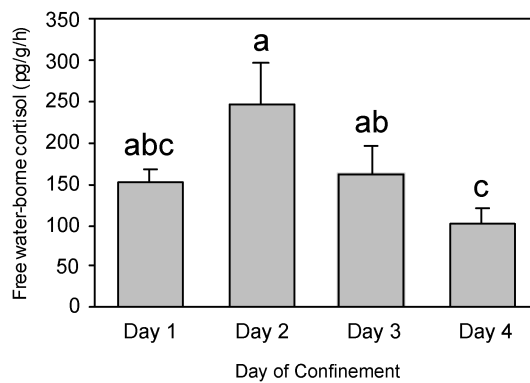


Figure 2. Change in free (ether extracted) water-borne cortisol release rates across four successive days of beaker confinement in male convict cichlids (Round 2). Histogram bars with different letter notations are significantly different after sequential Dunn–Sidak adjustment of least squares mean contrasts (day 2 vs. day 4: $p < 0.0001$; day 3 vs. day 4: $p = 0.02$; all other comparisons not significant, $p > 0.05$). True means \pm SEM rather than transformed means are shown.

Experiment 2: Handling stress

There was no significant overall effect of handling treatment on water-borne or plasma cortisol levels in either females (Round 1; water-borne: $F_{1,14} =$

0.82, $p = 0.38$; plasma: $F_{1,14} = 4.30$, $p = 0.06$) or males (Round 2; water-borne: $F_{1,23} = 3.04$, $p = 0.09$; plasma: $F_{1,19} = 0.32$, $p = 0.58$).

Experiment 2: Relationship between plasma and water-borne cortisol

There was a significant positive relationship between plasma (pg/ml) and water-borne (pg/g per h) cortisol (ANCOVA; $F_{1,18} = 9.66$, $p = 0.0061$; Figure 3), a pattern that held true regardless of treatment (no handling, handling) (see treatment \times plasma interaction under Statistical analyses).

Experiment 3: effects of exogenous cortisol

Cortisol release rates were influenced significantly by treatment ($F_{3,221} = 37.26$, $p < 0.0001$) and time ($F_{6,221} = 9.45$, $p < 0.0001$), and by the significant time \times treatment interaction ($F_{3,221} = 8.66$, $p < 0.0001$); these effects are described in the following sections. There were no significant differences in cortisol release rates within or among treatments during the pre-injection period (Days 1–3; linear contrasts with sequential Dunn–Sidak adjustments,

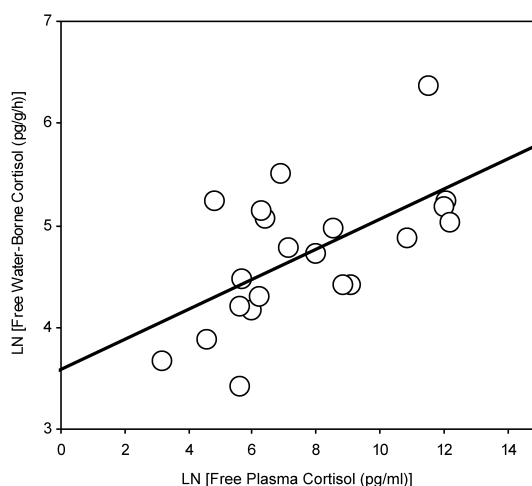


Figure 3. Significant positive relationship between plasma (pg/ml) and free (ether extracted) cortisol release rates (pg/g per h) in male convict cichlid fish from Experiment 2 (Round 2). Results from the analysis of covariance are described in the text; regression analysis was consistent with the ANCOVA ($F_{1,19} = 10.62$, $p = 0.0041$, $R^2 = 0.36$). The regression equation was $\ln(y) = 0.147 \ln(x) + 3.584$ where y = water release rate (pg/g per h) and x = plasma concentration (pg/ml).

$p \gg 0.05$), indicating that all animals were responding similarly to beaker confinement before manipulation.

Within-treatment patterns

The treatment \times time interaction (Figure 4) revealed a highly significant increase in cortisol at the 1 h post-injection time point for both CORTLO and CORTHI. Regarding the temporal pattern for CORTLO, one comparison remained highly significant after sequential Dunn–Sidak adjustments (1 h post-injection vs. pre-injection Day 3; linear contrast, $p < 0.0001$), indicating a marked but transient peak in cortisol caused by the treatment. CORTHI animals showed a more protracted cortisol peak that spanned the 1 h, 4 h and 24 h post-injection periods (1 h, 4 h, 24 h cortisol $>$ all other time points; linear contrasts, $p < 0.0001$). Individuals in SHAM showed virtually no variation across the time points. CNTRL animals showed a steady

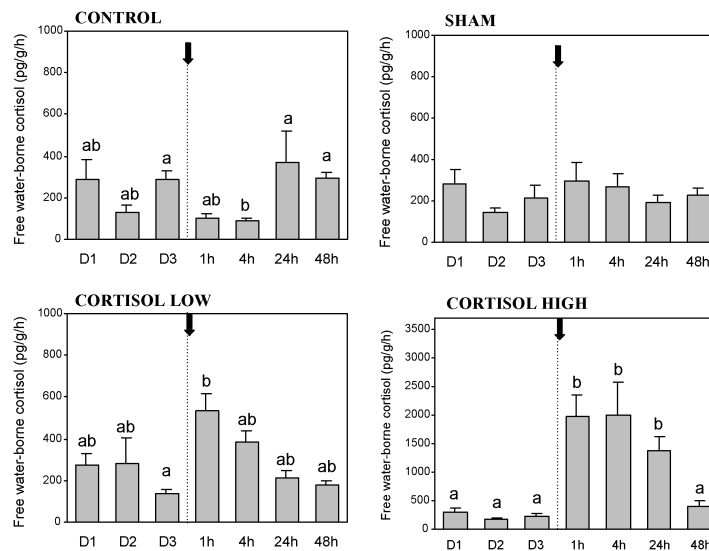


Figure 4. Temporal changes in free cortisol release rates (ethyl acetate eluted; mean \pm SEM, pg/g per h) both before and after treatment with only anesthetic (control), sesame oil injection (sham), or low and high doses of cortisol. Males and females are pooled for this analysis. Dashed lines coupled with a dark arrow denote the time of injection. Histogram bars labeled with different letters within each treatment are significantly different (after sequential Dunn–Sidak adjustments of the p values); graphs with no letter labels showed no significant temporal changes in cortisol release rates. See text for cross-treatment comparisons at each time point. All axes have the same scale except the ‘cortisol high’ treatment.

decrease from Day 1 to 4 h post-injection and then significant elevations in cortisol at the 24 h and 48 h points (linear contrasts, $p < 0.0006$).

Between-treatment patterns

At 1 h, 4 h and 24 h post-injection, CORTHI animals had significantly elevated cortisol release rates relative to animals in all other treatments (linear contrasts, $p < 0.0001$). CORTLO animals had significantly elevated cortisol release rates relative to CNTRL at both 1 h and 4 h post-injection (linear contrasts, $p < 0.0001$). At the final time point, 48 h, all treatment effects disappeared (linear contrasts, $p \gg 0.05$) (Figure 4).

Other patterns

There were no effects of sex ($F_{1,221} = 1.82$, $p = 0.18$), sex \times time ($F_{6,221} = 0.82$, $p = 0.56$), or treatment \times sex \times time ($F_{18,221} = 0.71$, $p = 0.80$), indicating that females and males showed parallel temporal changes in cortisol under all treatment conditions. Handling time (netting from home tank to beaker) had no significant effect on cortisol release rates ($F_{1,221} = 0.87$, $p = 0.35$). There was, however, a significant treatment \times sex interaction ($F_{3,221} = 4.54$, $p = 0.004$). This interaction was driven largely by females having significantly higher cortisol release rates than males in CNTRL (linear contrast, $p = 0.0005$; Figure 5) but, also by males being more sensitive overall to CORTLO (e.g., CORTLO vs. CNTRL, linear contrast, males: $p < 0.0001$; females: $p = 0.66$, Figure 5).

Experiment 3: Relationship between plasma and water-borne cortisol

On the final day (48 h), we obtained plasma from 23 of the 39 experimental animals across all treatments. Because animals in all treatments had returned to near baseline cortisol release rates at 48 h, we pooled the data across treatments to examine the correlation between plasma (pg/ml) and water-borne (pg/g per h) cortisol levels. There was a significant positive relationship between plasma cortisol concentrations and cortisol release rates into the water (linear regression: $F_{1,21} = 4.14$, $p = 0.05$; Figure 6).

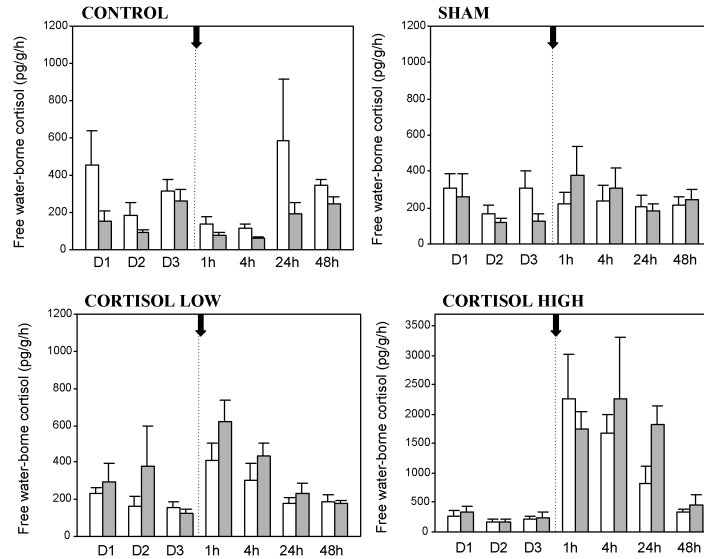


Figure 5. Temporal changes in free cortisol release rates (ethyl acetate eluted; mean \pm SEM, pg/g per h) both before and after treatment with only anesthetic (control), sesame oil injection (sham), or low and high doses of cortisol, differentiated by sex. Female cortisol patterns are shown in white bars and male cortisol patterns in gray bars. All axes have the same scale except the 'cortisol high' treatment.

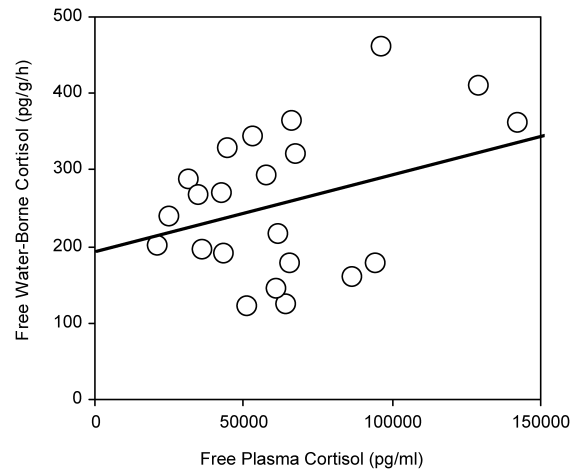


Figure 6. Significant positive relationship between the free cortisol fraction (ethyl acetate eluted) in plasma (pg/ml) and water (pg/g per h) in animals of Experiment 3. The regression equation is $y = 0.001x + 192.88$ where y = water release rates (pg/g per h) and x = plasma concentrations (pg/ml).

Discussion

Habituation to confinement

Convict cichlids mount a significant stress response upon first exposure to beaker confinement but they habituate to the beaker after 3–4 consecutive days of the same treatment. We found supporting but non-significant trends in the three days before injection of Experiment 3 (see D1, D2 and D3 in Figure 4). The lowest release rates of free cortisol across all experiments hovered consistently between 100 and 200 pg/g per h, regardless of extraction/elution method (e.g., ether extraction vs. ethyl acetate elution). These release rates correspond well with values inferred from studies on rainbow trout (Ellis et al., 2004; see Figure 5, no stress controls, bottom panel) and stickleback (Sebire et al., 2007; see Figure 6, bottom panel). Wysocki et al. (2006) reported similarly low cortisol concentrations for European perch, common carp, and gudgeons that were not exposed to ship noise, and Lower et al. (2005) found release rates of approx. 100 pg/g per h in common carp and roach prior to the implantation of a transmitter. Thus, it is reasonable to suggest that 100–200 pg/g per h represents the baseline cortisol release rates for convict cichlids, and that individuals of this species require 3–4 days of habituation to beaker confinement to achieve baseline levels.

It is important to note that some studies did not require habituation to achieve low cortisol release rates (Lower et al., 2005; Wysocki et al., 2006). In both of these studies, individual fish were housed in much larger quantities of water (relative to body size) than we used in this study, which perhaps alleviated confinement stress. Sebire et al. (2007), however, transferred sticklebacks to a small volume of water in a beaker (similar to the present study) and showed (see Figure 6, bottom panel, p. 35) a subtle trend for cortisol release rates to decline from the first to the third beaker exposure over a 6-day period. These results, coupled with our own, seem to suggest that habituation is necessary if the subjects will be placed in a small area (e.g., beaker) with a small volume of water relative to body size. In designing experiments, we are, thus, faced with a trade-off between (1) placing fish in large volumes, which then take significant time to extract and process and (2) using smaller volumes, which can be extracted rapidly but necessitate several days of habituation to confinement.

From a behavioral endocrinology perspective, we argue that it might be more informative to obtain both stress responsive (e.g., day 1 of confine-

ment; not habituated) and baseline (day 4 of confinement; habituated) cortisol release rates and, thus, to use the habituation design. In a previous study on convict cichlids, Earley et al. (2006) found that stress responsive cortisol levels (first exposure to a beaker) were positively correlated with the number of escalated aggressive acts performed during territorial contests, especially for eventual losers. Studies in rodents also have shown that rapid pulses of stress hormones can facilitate aggression (e.g., Mikics et al., 2004). On the other hand, Pottinger & Carrick (2001) demonstrated in rainbow trout that 'high stress responsive' animals tend to be subordinated by 'low stress responders'; however, they did not evaluate contest dynamics during the initial stages of fighting to determine whether the stress responsive lines differed in aggressive behavior. In contrast to stress responsive cortisol levels, naturally high (or augmented) baseline cortisol concentrations tend to reduce aggression and predict subordinate status in fishes (Sloman et al., 2001; Øverli et al., 2002; DiBattista et al., 2005; Earley & Hsu, 2008). In the future, we might be able to capitalize on the habituation protocol to obtain both stress responsive and baseline cortisol release rates from the same fish, which then can be used, for instance, to predict differences in individual behavior and/or success in aggressive contests.

Handling stress

A common method for inducing a cortisol response in fishes is to hold individuals out of water in a net for a short period of time. Ackerman et al. (2000) showed that 45 s of net holding was sufficient to trigger a significant increase in plasma cortisol in cutthroat trout (*Oncorhynchus clarki clarki*). In our experiments, we captured animals from their home tanks and held them out of water, immobile in a net, for 2–3 min. Our goal was to determine whether a relevant stressor would evoke a plasma cortisol response, and whether the predicted handling-induced elevations in plasma cortisol would be manifested as increased release rates over the gills. Net handling, however, did not elevate cortisol in either plasma or water-borne samples of convict cichlids. It is possible that routine netting and handling in the laboratory habituated the cichlids to this procedure. An alternative is that 30 min was too short an interval between net handling/emersion and plasma/water collection to detect a cortisol response. Ackerman et al. (2000) showed significant increased in plasma cortisol of handled versus unhandled fish at four

time points spanning 0.5 to 3 h after netting, with a peak difference at 1 h. Future studies should evaluate the temporal course of post-handling stress responses in convict cichlids, perhaps by assigning animals to treatments where water-borne hormones are collected for 30 min at 0, 0.5, 1 and 1.5 h after handling/emersion. It is worth noting that handled females tended to have higher cortisol release rates (445.1 ± 88.03 pg/g per h) and plasma cortisol concentrations (311.22 ± 61.00 ng/ml) than unhandled females (water release rates: 307.39 ± 35.5 pg/g per h; plasma concentrations: 184.98 ± 37.27 ng/ml); no discernable trend was evident in males. Thus, future studies might also investigate sex differences in cortisol responsiveness to acute stressors using a factorial design where females and males of similar size are tested concurrently, a design that we did not employ here. Indeed higher sensitivity of females to acute stressors appears to be the norm in rodent studies (Guimont & Wynne-Edwards, 2006) but is rarely considered in fishes.

Correspondence between plasma and water-borne cortisol

In two independent experiments (2 and 3), we demonstrated a significant positive relationship between plasma cortisol concentrations and water-borne cortisol release rates, indicating that free steroids liberated over the gills (see Ellis et al., 2005) of convict cichlids reflect circulating concentrations. The convict cichlid is the third species for which concordance between plasma and water-borne cortisol has been validated, rainbow trout and stickleback being the others (Ellis et al., 2004; Sebire et al., 2007). Interestingly, the plasma–water relationship that emerged from the handling experiment was curvilinear (ln–ln; Figure 3), while the relationship on the final day of the exogenous treatment experiment was linear (Figure 6). Sebire et al. (2007) found a curvilinear relationship in their study on stickleback. They hypothesized that the asymptote could be due to the inability of high plasma cortisol concentrations to diffuse effectively across the gills within the 30 min collection period. It is possible that we were limited in our ability to detect, within the 30 min collection time, a linear plasma–water relationship in male convict cichlids whose plasma levels were past some threshold (e.g., >100 ng/ml, $N = 5$ of 21 males in Experiment 2). Our second plasma–water correlation included animals whose cortisol release rates had returned to baseline (after injection or control treatment in Experiment 3). Individuals in this experiment showed much less variation in plasma cortisol concentrations and few surpassed 100 ng/ml. The resulting linear relationship provides support for the notion that the plasma–water correlation can be, in part, con-

strained by the interaction between plasma concentrations and time allotted for diffusion across the gills to occur. In this second case, 30 min appeared sufficient to maintain a linear relationship.

Exogenous treatment with cortisol

Exogenous administration of steroid hormones effectively alters plasma endocrine profiles and associated behavioral output (e.g., Øverli et al., 2002; DiBattista et al., 2005; Remage-Healey & Bass, 2006). We hypothesized that exogenous manipulation of the stress axis by cortisol injection would increase cortisol release rates into the water relative to baseline and control conditions. Our results clearly demonstrate that injection of low- or high-dose cortisol induces significantly greater release rates than baseline (Day 3) measures in the same individual, and relative to animals in the control and sham conditions. Peak cortisol release rates were evident as soon as 1h post-injection. The high dose elicited a stronger (approx. 3.5–4-fold) and more persistent water-borne cortisol peak than the low dose. These results strongly suggest that release rates into the water mirror changes, even if exogenously induced, in the endocrine state of convict cichlid fish. Presumably, injection of cortisol caused significant, acute increases in circulating stress hormone levels, which were released into the surrounding water via diffusion through the gills (Ellis et al., 2005). Future experiments that administer various doses of cortisol, or other steroid hormones, and employ different times for beaker occupancy perhaps could resolve the aforementioned issue of release rates being constrained both by time spent in the beaker and plasma steroid concentrations. In addition, future studies interested in validating the water-borne hormone collection technique also should challenge individuals with injection of hypothalamic or pituitary hormones (e.g., LHRH, ACTH) known to elicit the release of focal hormones such as cortisol, testosterone and 11-ketotestosterone (Hirschenhauser et al., 2004; Bshary et al., 2007; Scott et al., 2008). Challenges with peptide hormones upstream of steroid synthesis coupled with rigorous temporal analyses and both plasma and water sampling will provide greater insights into how well water-borne hormones reflect the endocrine status of fishes.

Acknowledgements

We would like to extend our sincere thanks to Sandy Scott, Eduardo Barata, Peter Hubbard and Adelino Canario for organizing a stimulating workshop on Bioactive Water Borne Chem-

icals and to the Center of Marine Sciences for providing the facilities for the event. We also are grateful to two anonymous reviewers, Matthew Grober, Edmund Rodgers, Varenka Lorenzi, Michael Black, Julie Desjardins, Yuying Hsu, Luke Remage-Healey and all workshop participants for insightful comments and discussions on the water-borne hormone method. Lastly, we thank Alex Cheah, Donald Copeland, Heidi Dunbar, Whitney Janzen, Jacqueline Ma, Boopathy Sivaraman and Haley Stephenson for experimental assistance and help with animal maintenance.

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