

Development of a modified cortisol extraction procedure for intermediately sized fish not amenable to whole-body or plasma extraction methods

Taylor W. Guest · Reginald B. Blaylock ·
Andrew N. Evans

Received: 11 May 2015 / Accepted: 29 July 2015 / Published online: 6 August 2015
© Springer Science+Business Media Dordrecht 2015

Abstract The corticosteroid hormone cortisol is the central mediator of the teleost stress response. Therefore, the accurate quantification of cortisol in teleost fishes is a vital tool for addressing fundamental questions about an animal's physiological response to environmental stressors. Conventional steroid extraction methods using plasma or whole-body homogenates, however, are inefficient within an intermediate size range of fish that are too small for phlebotomy and too large for whole-body steroid extractions. To assess the potential effects of hatchery-induced stress on survival of fingerling hatchery-reared Spotted Seatrout (*Cynoscion nebulosus*), we developed a novel extraction procedure for measuring cortisol in intermediately sized fish (50–100 mm in length) that are not amenable to standard cortisol extraction methods. By excising a standardized portion of the caudal peduncle, this tissue extraction procedure allows for a small portion of a larger fish to be sampled for cortisol, while minimizing the potential interference from lipids that may be extracted using whole-body homogenization

procedures. Assay precision was comparable to published plasma and whole-body extraction procedures, and cortisol quantification over a wide range of sample dilutions displayed parallelism versus assay standards. Intra-assay %CV was 8.54 %, and average recovery of spiked samples was 102 %. Also, tissue cortisol levels quantified using this method increase 30 min after handling stress and are significantly correlated with blood values. We conclude that this modified cortisol extraction procedure provides an excellent alternative to plasma and whole-body extraction procedures for intermediately sized fish, and will facilitate the efficient assessment of cortisol in a variety of situations ranging from basic laboratory research to industrial and field-based environmental health applications.

Keywords Cortisol extraction · Fish physiology · Aquaculture · Spotted Seatrout · Stress response · Stock enhancement

Introduction

The physiological stress response is an adaptive mechanism that enhances short-term survival in large part by mobilizing energy resources to facilitate the cellular and organismal response to physical threats or environmental challenges. The most widely used indicator of stress in fish is the steroid hormone cortisol, which increases in response to various physical stressors and mediates elevated plasma levels of

T. W. Guest (✉) · R. B. Blaylock · A. N. Evans
Gulf Coast Research Laboratory, Department of Coastal
Sciences, University of Southern Mississippi, 703 East
Beach Drive, Ocean Springs, MS, USA
e-mail: taylorguest@msn.com

R. B. Blaylock
e-mail: reg.blaylock@usm.edu

A. N. Evans
e-mail: andrew.n.evans@usm.edu

oxidative fuels such as glucose (Mesa 1994; Wendelaar Bonga 1997; Mommsen et al. 1999; Ramsay et al. 2006; Barcellos et al. 2007; and Ramsay et al. 2009). However, in addition to short-term adaptive effects, chronic elevation of cortisol can be deleterious to the overall health of the animal through suppression of critical physiological systems including growth, reproduction, and the immune response (Barton 2002). Therefore, the quantification of cortisol is important both for studies examining the stress physiology of fishes and for studies or programs (e.g., aquaculture) interested in the assessment or long-term monitoring of fish health. The most common method for cortisol quantification is the extraction of steroid hormones from plasma or serum followed by a specific radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (EIA) (Barton et al. 1987; Flos et al. 1988; Avella et al. 1991; Iwama et al. 1992; Pankhurst and Dedual 1994; Barton and Zitzow 1995; Iwama et al. 1997; and Barnett and Pankhurst 1998). However, the total blood volume required for efficient plasma cortisol analysis via RIA or EIA is approximately 50–150 μl (Sink et al. 2007), which is difficult or impossible to obtain from small teleost species and/or larval and juvenile stages. Also, blood samples cannot be taken post hoc from samples originally collected for other purposes, or in situations in which time is limited and fish must be frozen for subsequent analyses. A more recently developed method for quantifying cortisol in such small fish (200–750 mg) is whole-body homogenization followed by lipid (including steroid hormone) extraction (Ramsay et al. 2006; Sink et al. 2007; Peterson and Booth 2009; Yeh et al. 2013). There are currently no published extraction methods for the quantification of cortisol in intermediate size classes, i.e., fish that are too small to provide an adequate plasma sample and too large for efficient whole-body homogenization and extraction. These intermediately sized subjects require considerable laboratory resources for whole-body extraction, and also present the problem of inefficient homogenization due to the presence of bone and large organs as well as binding interference within the assay due to excess lipids. More specifically, interfering substances such as nontarget cholesterol-based hormones and various lipids within the sample tissue, found in significant quantities in larger subjects, can cause inconsistency when using whole-body extraction methods. Therefore, for intermediately sized fish a technique that is reproducible,

reduces resource use, requires a smaller sample, and alleviates the problems with lipid interference encountered using whole-body extraction is required.

The Gulf Coast Research Laboratory releases hatchery-reared juvenile Spotted Seatrout (*Cynoscion nebulosus*) to study the effectiveness of stock enhancement, and is actively conducting research that attempts to improve stock enhancement procedures and outcomes. However, due to the intermediate size range of released fish (50–100 mm) and rapid pace of release procedures, conventional cortisol extraction procedures limit the use of cortisol as a measure of the effect of hatchery and release procedures on the fish. Therefore, in this study we developed a novel steroid extraction procedure to address this gap in our ability to quantify cortisol in fish within an intermediate size range. The use of a discrete section of the caudal peduncle facilitates accuracy and reproducibility by eliminating the homogenization of internal organs and a large amount of bone, as well as reducing the amount of total lipids within the homogenate. We demonstrate that this extraction procedure allows for efficient, accurate, and repeatable cortisol quantification in an intermediate size class of fish, i.e., 50–100 mm in length and 1–13 g in weight.

Methods

Fish

For assay development and validation, unstressed Spotted Seatrout ($n = 14$) were acquired from the Thad Cochran Marine Aquaculture Center at the Gulf Coast Research Laboratory in Ocean Springs, Mississippi. Seatrout were laboratory-reared from captive adult broodstock, and ranged in size from 50 to 100 mm in length and 1–13 g in weight (age: 48–80 days post-hatch). Following collection by net, whole fish were immediately frozen individually on dry ice and then stored at $-80\text{ }^{\circ}\text{C}$ until the cortisol extraction procedure. Larger individuals amenable to phlebotomy (age: 115 days post-hatch; length: ~ 125 mm; $n = 18$) were used for the comparison of changes in tissue versus plasma cortisol in response to stress, as described below.

Cortisol extraction procedure and EIA

For cortisol extraction, whole fish were partially thawed and weighed (g) prior to processing to allow

for determination of cortisol concentration per unit of mass following extraction and EIA. After weighing, a segment of tissue from the posterior end of the dorsal fin to the anterior end of the caudal fin was removed from each fish (Fig. 1). We selected this region because it is primarily muscle and located in an area with clear demarcations (caudal and dorsal fins) that facilitate consistent sampling of the same section between individuals. Each excised caudal peduncle segment was then weighed, diced into smaller sections, and placed into a clean test tube containing 2 mL of ice cold 1X phosphate-buffered saline (PBS, pH 7.4). Samples were homogenized for 30 s using a Tissue-Tearor (Biospec Products) at the maximum setting. Resulting homogenates were transferred to clean, screw-cap (16 mL) test tubes. The tubes used during the homogenization process were rinsed twice with 5 mL of diethyl ether (BDH Chemicals) and decanted into the screw-cap test tubes. The screw-cap test tubes were then capped and vortexed for 1 min using a Mini Vortex Mixer (Fisher Scientific). Vortexed samples were kept on ice until each sample was completed. Samples were then centrifuged at $2000\times g$ for 5 min using an Allegra[®] X-15R (Beckman Coulter). Following centrifugation, the diethyl ether phase was collected from each sample using Pasteur pipettes and placed into individual test tubes on ice. The above procedure was performed twice for each sample to ensure maximum cortisol extraction. The pooled diethyl ether fraction from the two extractions for each sample was then dried under a stream of nitrogen at 42 °C. Dried samples were reconstituted in 500 μ L of assay buffer, sealed in test tubes with parafilm and stored at 4 °C, typically overnight, but never more than 96 h (Ramsay et al. 2006). Two 50 μ L aliquots of each reconstituted sample were taken from the 500 μ L sample stock and used in duplicate wells following the assay protocol provided by Cayman Chemical (Cortisol EIA Kit, Cayman Chemical Item Number 500360). The standard published kit protocol was used throughout the



Fig. 1 Image of a Spotted Seatrout. The red box indicates the region of the caudal peduncle used for the tissue extraction procedure

plate validation assay to ensure optimal results. Samples were quantified using a SpectraMax M2 microplate reader (Molecular Devices) and an absorbance of 405 nm.

Assay validation

Steroid recovery

Six additional fish were used to determine the efficiency of steroid recovery using the described tissue extraction procedure. Each fish was partially thawed and weighed, with the caudal peduncle portion subsequently excised and processed as described above. A 1 mL sample was taken from each of the six homogenates to serve as the control (unspiked) sample. Another 1 mL sample (spiked sample) from each homogenate was then spiked with 800 pg of cortisol (Cayman Chemical). Both control and spiked homogenates were then processed using the described method with cortisol subsequently quantified using the Cayman cortisol EIA to calculate the extraction efficiency of the procedure.

Dilution series

A parallelism test was used to determine whether cortisol values were consistent over a wide range of sample dilutions, to assess both the useful range of the assay and the potential for interference of other lipids with antibody binding at high or low concentrations of homogenate. Three fish were used to assess parallelism by diluting homogenate in kit-specific EIA buffer using the following series: undiluted, 1:2, 1:4, 1:8, and 1:16. Samples were then extracted as described above and cortisol quantified using the Cayman Chemical EIA. For direct comparison, a dilution series using the highest standard (4000 pg mL^{-1}) from the Cayman Chemical EIA was included using the following series: undiluted, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256.

Cortisol and handling stress

A comparative study was conducted to assess changes in plasma and tissue cortisol in response to standard hatchery procedures. To determine whether changes in plasma cortisol are correlated with changes in tissue values using the described method, larger fish amenable to phlebotomy (age: 115 days post-hatch; length: ~ 125 mm) were used and both blood and tissue

samples were collected from the same fish. Approximately 20 fish were rapidly dipnetted from a single growout tank, with ten individuals sampled within 5–6 min to represent basal cortisol values. The remaining fish were then transferred to a separate tank and sampled after 30 min ($n = 10$). For each fish, approximately 100 μL of blood was collected from the caudal vessel just posterior to the anal fin using a heparinized syringe and 21-gauge needle, and then, each fish was immediately placed in a labeled ziplock bag on dry ice and stored at $-80\text{ }^{\circ}\text{C}$ overnight. Whole blood was transferred to a 0.5-mL microcentrifuge tube, centrifuged at $5000\times g$ for 5 min, and then, plasma was transferred to a separate microcentrifuge tube and stored at $-20\text{ }^{\circ}\text{C}$ overnight. Plasma cortisol was extracted by adding $5\times$ volume of diethyl ether, followed by centrifugation at $2000\times g$ for 5 min. Extractions were conducted twice for each sample to maximize extraction efficiency, and then, samples were dried under nitrogen and reconstituted in assay buffer before cortisol quantification following the manufacturer's instructions.

Statistical analyses

Standardized sample values for the standard curve, linearity of dilutions, and the coefficient of variation (CV) of unknown and spiked samples were determined from $\%B/B_0$ values and a 4-parameter logistic fit performed using the Cayman Chemical data analysis service MyAssays (myassays.com). Recovery of spiked samples and intra-assay percent CV were calculated independently. Pearson's correlation coefficient was used to assess the relationship between plasma and tissue cortisol values for control and stressed fish.

Results

Results for the tissue extraction procedure and subsequent validation assays demonstrate precision between duplicate wells and precision among replicated samples for both spiked and unspiked samples. Intra-assay percent CV was 8.54 %. All sample dilutions show good parallelism compared with the cortisol standard dilution series (Fig. 2). Results from the comparison of spiked and unspiked samples demonstrate an average recovery of 102 % with a range of averages from 92.08 pg mL^{-1} cortisol in unspiked samples to 914.95 pg mL^{-1} in spiked samples. Percent CV for

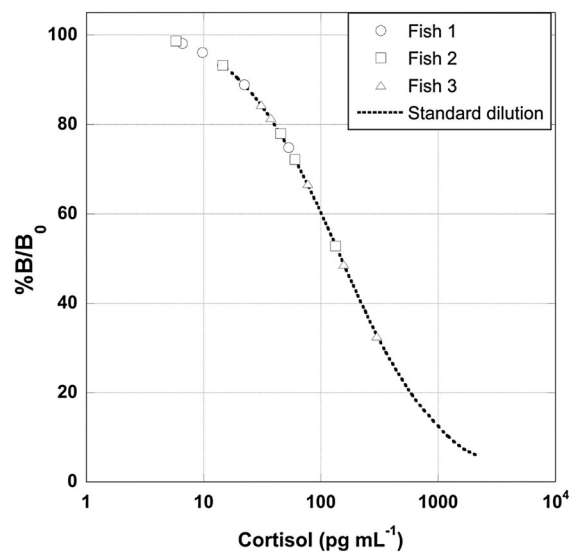


Fig. 2 Dilution series confirming parallelism for the tissue extraction procedure. The *dotted line* represents a twofold dilution series using the highest cortisol standard (4000 pg mL^{-1}) from the Cayman Chemical EIA. Following extraction, samples from three control fish were diluted twofold four times (undiluted, 1:2, 1:4, 1:8, and 1:16). All concentrations are in pg/mL

unspiked and spiked samples was 4.17 and 2.91 %, respectively (Fig. 3). Following handling stress, cortisol increased significantly in both plasma ($p < 0.001$) and tissue ($p < 0.001$) versus control values, with a greater increase in average plasma cortisol levels (control = $11,782\text{ pg mL}^{-1}$, stress = $25,905\text{ pg mL}^{-1}$) than in tissue cortisol levels (control = 4230 pg g^{-1} , stress = 5083 pg g^{-1}). Plasma and tissue cortisol values were significantly correlated (Pearson's correlation coefficient $R = 0.7445$, $p = 0.0004$; Fig. 4).

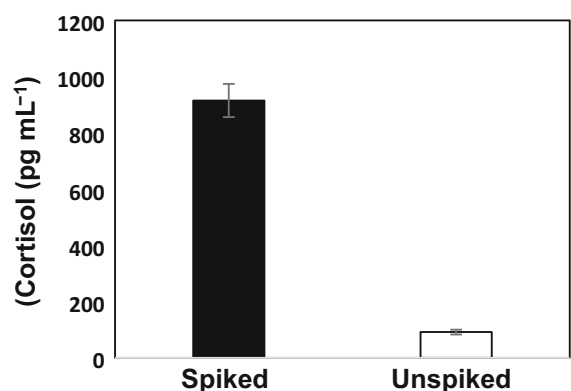


Fig. 3 Cortisol values for control (unspiked) samples versus samples spiked with 800 pg cortisol prior to extraction ($n = 6$)

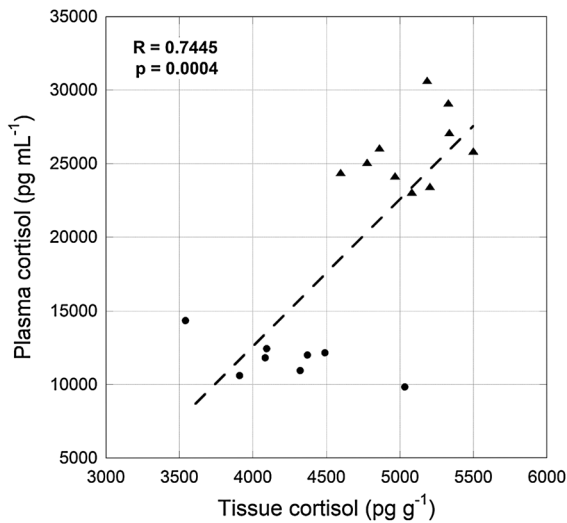


Fig. 4 Linear regression of plasma (pg mL^{-1}) versus tissue (pg g^{-1}) cortisol values for control fish (circles, $n = 8$) and experimental fish 30 min after handling stress (triangles, $n = 10$)

Discussion

The goal of this study was to develop a cortisol extraction procedure that facilitates the accurate and efficient quantification of cortisol concentrations in individual fish not amenable to standard cortisol extraction procedures. Our results demonstrate that excision of a defined portion of the caudal peduncle followed by homogenization and steroid extraction provides cortisol measurements within reported basal values for other species, with excellent cortisol extraction efficiency and consistency between individuals and assays. Whole-body extraction procedures not only extract cortisol and other steroid hormones, but also additional lipids within the sample tissue. Sink et al. (2007) noted that reconstitution of the dried sample from a whole-body extraction with an aqueous solution, such as PBS, does not allow the final product to be properly emulsified due to the large amount of nontarget lipids extracted during the whole-body extraction procedure. While nontarget lipids also were co-extracted using the current procedure, the selection of a discrete region of the caudal peduncle minimizes the extraction of lipids from major organs and tissues, e.g., lipid-rich adipose and hepatic tissue.

While it is not expected that cortisol values from the selected region represent average per gram values for the entire fish (i.e., cortisol is not distributed evenly

throughout the body), basal cortisol values from 50 to 100 mm *Cynoscion nebulosus* in the current study ranged from 0.80 to 2.4 ng g^{-1} , and values using whole-body extraction from multiple species ranged from 0.50 to 8.0 ng g^{-1} . This consistency with published values from whole-body extractions therefore may allow for the comparison of cortisol values obtained using whole-body extraction methods and this selected-tissue extraction method, although further study is needed to validate such a comparison within species of interest including *C. nebulosus*. Also, in larger *C. nebulosus* individuals tissue values quantified using this method were significantly correlated with plasma cortisol in control fish versus those subjected to moderate handling stress typical in a hatchery environment, even over a relatively short period of time (30 min). While changes in tissue cortisol ($\sim 20\%$ increase) were not as great as changes in plasma values ($\sim 120\%$ increase), the difference in control versus stressed tissue cortisol values was highly significant, validating the use of this method for assessing the general health of fish as well as the impacts of putative stressors. The lower proportional change in tissue versus plasma cortisol following stress may reflect the relatively low blood content of the section of peduncle sampled, which was specifically selected as a homogenous region composed primarily of muscular tissue for consistency. The consistent basal cortisol values obtained by sampling this tissue from all size ranges evaluated in this study, combined with significant changes in cortisol 30 min after a handling stressor, support the use of this method for assessing cortisol values in fish that are not amenable to more traditional methods due to either size or other experimental or logistical constraints. Indeed, we have successfully used this method to demonstrate changes in tissue cortisol of 50–100 mm *C. nebulosus* in response to specific handling and transport procedures involved in the release of hatchery fish to the wild (manuscript in preparation). In conclusion, the described procedure will allow researchers to consistently sample intermediately sized fish that are too large for whole-body extraction procedures, but too small to draw blood for plasma or serum steroid extraction. Additional valuable aspects of the described extraction procedure include ease of use, repeatability, and efficient use of laboratory resources versus whole-body extractions, which may therefore facilitate the quantification of

cortisol in large-scale field monitoring programs, high-volume aquaculture facilities, the seafood industry, or other areas where traditional methods are suboptimal or cannot otherwise be performed.

Acknowledgments We thank the Thad Cochran Marine Aquaculture staff for help with fish rearing and care. This project was funded by a Gulf of Mexico Energy Security Act (GOMESA) grant from the Mississippi Department of Marine Resources and the Mississippi Department of Marine Resources Tidelands Trust Fund Program.

References

- Avella M, Schreck CB, Prunet P (1991) Plasma prolactin and cortisol concentrations of stressed coho salmon, *Oncorhynchus kisutch*, in fresh water or salt water. *Gen Comp Endocrinol* 81:21–27
- Barcellos LJG, Ritter F, Kreutz LC, Quevedo RM, Silva LB (2007) Whole-body cortisol increases after direct and visual contact with a predator in Zebrafish, *Danio rerio*. *Aquaculture* 272:774–778
- Barnett CW, Pankhurst NW (1998) The effects of common laboratory and husbandry practices on the stress response of greenback flounder, *Rhombosolea tapirina*. *Aquaculture* 162:313–329
- Barton BA (2002) Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integr Comp Biol* 42:517–525
- Barton BA, Zitzow RE (1995) Physiological responses of juvenile walleyes to handling stress with recovery in saline water. *Progress Fish-Cultur* 57:267–276
- Barton BA, Schreck CB, Barton LD (1987) Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions, and stress responses in juvenile rainbow trout. *Dis Aquat Organ* 2:173–185
- Flos R, Reig L, Torres P, Tort L (1988) Primary and secondary stress responses to grading and hauling in rainbow trout, *Salmo gairdneri*. *Aquaculture* 71:99–106
- Iwama GK, McGeer JC, Bernier NJ (1992) The effects of stock and rearing density on the stress response in juvenile coho salmon (*Oncorhynchus kisutch*). *ICES Mar Sci Symp* 194:67–83
- Iwama GK, Pickering AD, Sumpter JP, and Schreck CB (1997) Fish stress and health in aquaculture. Society of experimental biology seminar series 62
- Mesa MG (1994) Effects of multiple acute stressors on the predator avoidance ability and physiology of juvenile Chinook salmon. *Trans Am Fish Soc* 123:786–793
- Mommsen TP, Vijayan MM, Moon TW (1999) Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev Fish Biol Fisheries* 9:211–268
- Pankhurst NW, Dedual M (1994) Effects of capture and recovery on plasma levels of cortisol, lactate and gonadal steroids in a natural population of rainbow trout. *J Fish Biol* 45:1013–1025
- Peterson BC, Booth NJ (2009) Validation of a whole-body cortisol extraction procedure for channel catfish (*Ictalurus punctatus*) fry. *Fish Physiol Biochem* 36:661–665
- Ramsay J, Feist G, Varga Z, Westerfield M, Kent M, Schreck CB (2006) Whole-body cortisol as indicator of crowding stress in adult Zebrafish, *Danio rerio*. *Aquaculture* 258:565–574
- Ramsay J, Feist G, Varga Z, Westerfield M, Kent M, Schreck CB (2009) Whole-body cortisol response of Zebrafish to acute net handling stress. *Aquaculture* 297:157–162
- Sink TD, Kumaran S, Lochmann RT (2007) Development of a whole-body cortisol extraction procedure for determination of stress in golden shiners, *Notemigonus crysoleucas*. *Fish Physiol Biochem* 33:189–193
- Wendelaar Bonga SE (1997) The stress response in fish. *Physiology Reviews* 77:591–625
- Yeh CM, Glöck M, Ryu S (2013) An optimized whole-body cortisol quantification method for assessing stress levels in larval Zebrafish. *PLoS ONE* 8:e79406. doi:[10.1371/journal.pone.0079406](https://doi.org/10.1371/journal.pone.0079406)