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Adaptation and validation of a radioimmunoassay kit for measuring plasma cortisol in turbot

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Abstract

Levels of cortisol in fish blood provide quantitative information on the degree of stress induced by a variety of stressors. It is also useful in describing the social status of individual fish within groups. The commercial production of radioimmunoassay (RIA) kits, such as the DPC® Coat-A-Count radioimmunoassay kit, has considerably reduced the effort required for cortisol measurement. These kits employ human plasma based cortisol standards which are not compatible for use with non mammalian species such as fish e.g. turbot, *Scophthalmus maximus* (Rafinesque), blood due to the interference effect of lipids and steroid binding proteins present in the plasma. In this study the DPC® kit was used following the removal of these lipids and steroid binding proteins from the plasma using an ethanol–hexane extraction. Excessive variability in the cortisol values obtained using this method deemed it unsatisfactory in overcoming the problem of incompatibility. A second modification of this technique that was tested involved the preparation of turbot specific standards for use in the preparation of modified standard curves. Using this method, an accuracy of 93.4% was achieved, as opposed to 79.6% using the kit human plasma based standards, and 47.1% using samples following lipid removal using an ethanol–hexane extraction. Based on analysis of accuracy, precision and reproducibility it is concluded that commercially available cortisol kits are suitable for use with turbot plasma, but a number of minor modifications are necessary.

Keywords: Cortisol; Plasma; Radioimmunoassay; Stress; Turbot

1. Introduction

Stress in fish resulting from husbandry practices (handling, crowding, transport etc.) can have detrimental effects on the health status of fish, resulting, at worst, in mortalities. It is advantageous to be able to quantify the effects of these practices on fish populations, at a sub-lethal level [16]. The stress response in teleost fish is non-specific qualitatively, encompasses a wide array of physiological systems and can be divided into primary and secondary effects [10]. The primary stress response is an endocrine one resulting in elevated levels of circulating catecholamines (adrenaline and noradrenaline) and corticosteroids (primarily cortisol). These induce secondary, longer lived stress responses such as altered circulating glucose levels [10]. Investigations of the endocrine stress response in teleost fish have focused mainly on the Hypothalamo–Pituitary–Interrenal (HPI axis) response [3,12,13].

While catecholamine changes in response to stress are of relatively short duration, corticosteroid elevation is more prolonged. Corticosteroids also demonstrate a response latency, which allows for distinction between sampling effects and pre-capture hormone levels [12].

Cortisol is not stored, but synthesised from cholesterol in the interrenal gland of teleost fish before being released into the blood stream [7]. Cortisol synthesis is regulated by the secretion of adrenocorticotrophic hormone from the *pars distalis* of the pituitary gland, which, in turn is controlled by the secretion of an, as yet unidentified, corticotrophin releasing factor from the hypothalamus. Functions include regulation of car-

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bohydrate and protein metabolism, growth, osmoregulation, and hence seawater adaptation [14]. In response to a non-specific stressor, the activation of the HPI axis results in increased secretion of corticosteroids, primarily cortisol [18], sometimes referred to as cortisolemia [15]. Additionally, circulating cortisol levels are indicative of the social and reproductive status of an individual fish and on the stability of it's social situation [7]. In humans, cortisol secretion shows strong diel variation. Such natural diel variation, although common in a number of fish species [19], is absent in turbot [20].

Plasma cortisol levels can be measured by a number of methods including HPLC with UV detection, radioimmunoassay (RIA) [8], isotope dilution gas chromatography-mass spectrometry [6] and ELISA [2]. Recent developments in RIA techniques have resulted in the production of commercially available kits which reduce the effort required for cortisol measurement. Commercially available cortisol kits have been successfully used to measure cortisol levels in a number of fish species, including channel catfish, *Ictalurus punctatus*, [1] mackerel, *Trachurus japonicus*, and sea bream, *Pagrus major*, [5]. However, these kits employ human plasma based standards not always suitable for comparison with fish plasma.

Lipids and steroid binding proteins present in turbot blood have been shown to exert a negative influence on RIA performance for plasma cortisol in turbot [20]. Such interference has not been observed in other species e.g. salmon, *Salmo salar*, [19]. Preliminary investigations carried out in this laboratory showed a 70% recovery of exogenous cortisol from turbot plasma, using a human plasma based RIA method, indicating that extraction of this interference may be necessary in order to accurately measure cortisol levels in turbot plasma using an RIA kit.

The growing commercial importance of aquaculture in Europe in recent years has lead to a need for an in depth understanding of individual species biology as it relates to culture. Of prime importance is the understanding of the stress induced by a variety of husbandry practices. Investigation of the stress response associated with handling procedures is made possible by the availability of a simple method for cortisol estimation. In consideration of these facts, this study set out to adapt and validate a commercially available RIA kit to measure plasma cortisol levels in turbot, thus providing a technique for use in physiological studies of the stress response of this species.

2. Materials and methods

2.1. Experimental environment

Juvenile turbot, *Scophthalmus maximus* (Rafinesque), obtained from a commercial turbot hatchery, were reared in circular tanks in a recirculation unit at the Aquaculture Development Centre, University College, Cork, Ireland for six months prior to experimentation. During this time they were fed to satiation three times daily. The mean temperature ($\pm$S.D.) over the six month period was 20.2 ± 2.3°C, and the photoperiod regime was 12:12 h (light:dark). The mean wet weight ($\pm$S.D.) of the experimental fish was 139.5 ± 43.6 g.

2.2. Collection of samples

All fish were starved for 24 h prior to sampling. In order to obtain blood samples from stressed turbot, fish were confined under water in nets for ten minutes and blood samples taken 60 min after initiation of the recovery period. Peak cortisol levels in turbot plasma are reportedly observed approximately 60 min after administration of this stressor [20]. Both stressed and unstressed turbot were removed from the tank individually and anaesthetised using Benzocaine before sampling. Blood samples were collected using a 1 ml syringe and a 21 gauge, 2.5 cm needle from the caudal vessel. Blood was immediately placed in 2 ml Lithium heparin tubes and centrifuged at $3\,500 \times g$ for 7 min. Following centrifugation plasma was aspirated off and stored at -70°C prior to analysis.

2.3. Cortisol analysis

The commercially available competitive binding Coat-A-Count® cortisol kit used was obtained from Diagnostic Products, California, Inc. This is a solid-phase RIA designed for quantitative measurement of cortisol in human plasma. Endogenous cortisol was stripped from pooled samples of turbot plasma by the method of Mitsuma [11]. A total of 200 mg of Norit-A charcoal was added to each 1 ml of turbot plasma and mixed for 24 h at 4°C. The resulting slurry was centrifuged at $12\,500 \times g$ for 120 min at 4°C and the supernatant filtered twice through a Millipore 0.2 μm filter. A stock cortisol standard was prepared by adding 10 mg of cortisol (Sigma Chemicals, UK) to 10 ml of ethanol giving a concentration of 2.76 mmol l^{-1}. This was further diluted in a PBS buffer to give a working standard of 5517.2 nmol l^{-1}. This standard was then added to charcoal stripped turbot plasma to prepare turbot specific cortisol standards ranging in concentration from 7.8–501.6 nmol l^{-1}.

2.4. Extraction of lipids

In order to determine whether extraction of interfering lipids and steroid binding proteins from turbot plasma was necessary prior to use with this kit an ethanol–hexane extraction of steroids and lipids was

carried out following the method of Waring [20]. Solvent extraction strips steroid off binding proteins in addition to preferentially solubilising free steroid. 600 μl of ultrapure ethanol was added to 75 μl of sample and centrifuged at 15 000 × *g* for 3 min. A volume of 600 μl of supernatant was diluted to a 70% solution by the addition of 250 μl of distilled water. A volume of 500 μl of hexane was added and the mixture mechanically shaken for 60 s and left to stand at room temperature (r.t.) for 15 min to allow the phases to separate. The upper hexane layer was aspirated off and discarded. A volume of 700 μl of the ethanol layer was dried and the cortisol resuspended in 800 μl 0.01 M phosphosaline buffer containing 1% bovine serum albumin (pH 7.5). The resulting samples were left overnight at 4°C prior to analysis.

2.5. Sample analysis

Turbot specific cortisol standards were made by preparing known concentrations of cortisol in charcoal stripped turbot plasma to give standard concentrations of 501.6, 250.8, 125.4, 62.7, 31.4, 15.7 and 7.8 nmol l^{-1}. All samples to be analysed, including standards and controls were assayed in duplicate. In the assay, 50 μl of each sample to be assayed was pipetted into the Ab-Coated tubes and 1.0 ml of ^{125}I Cortisol added. The tubes were then incubated for 45 min at 37°C in a water bath. The contents of all tubes were decanted, and allowed to drain for 5 min before being read on a gamma counter for 1 min. A calibration curve was constructed on logit-log graph paper and used to convert results from percent binding cortisol to concentration (nmol l^{-1}). The kit instructions recommend using 25 μl of each sample in the initial stage, due to the ease with which larger blood samples (circa 1 ml) can be obtained from juvenile turbot, this value was doubled in an attempt to improve the sensitivity of the assay. Known concentrations of cortisol in charcoal stripped plasma were assayed both directly and following ethanol extraction. The concentrations used were 1253.9, 627.0, 313.5, 156.7 and 78.4 nmol l^{-1}.

2.6. Data analysis

The accuracy of the assay was determined by the percentage recovery of the exogenous cortisol from pooled samples of charcoal stripped plasma spiked with known concentrations of cortisol. Parallelism was determined by serially diluting turbot plasma samples, to which known concentrations of cortisol had been added, in charcoal stripped plasma. Dilution ratios used were 1:2, 1:4, 1:8 and 1:16. Each sample dilution series was plotted and the shapes of the resulting curves were compared. Reproducibility was determined using the interassay variability (CV) between samples measured in a number of assays. Precision of the assay was calculated by determining the intra-assay variability (CV) of repeated measures of samples in one assay. Sensitivity of the assay was determined by calculating the least amount of cortisol distinguishable from zero as 2.5 times the S.D. of repeated measures of zero samples in one assay.

Data are reported as mean ± S.D. The coefficient of variation (CV) was calculated according to the formula

$$CV = (S.D./\bar{x}) \times 100.$$

Differences between means were analysed using a student's *t*-test. Differences in the slopes of the serial dilution plots were tested using an unpaired *t*-test. Differences at the 0.05% level were considered significant. All analyses were carried out using SPSS for Windows (Release 8.0).

3. Results

The curve obtained using known cortisol concentrations serially diluted in charcoal stripped turbot plasma was parallel to the standard curve obtained using standards provided with the kit (Fig. 1). The curve obtained using known concentrations of cortisol in stripped turbot plasma following ethanol extraction, however, was not as precise a fit as that observed without ethanol–hexane extraction.

The accuracy of the assay, calculated as percent recovery of exogenous cortisol from spiked charcoal stripped plasma was lower following ethanol extraction (Table 1). The percent recovery without ethanol extraction (79.6%, $n = 5$) was significantly higher than recovery following ethanol extraction (47.1% $n = 5$) ($t = 5.81$, d.f. = 8, $P < 0.01$).

A calibration curve was constructed based on the turbot specific standards against which all samples were

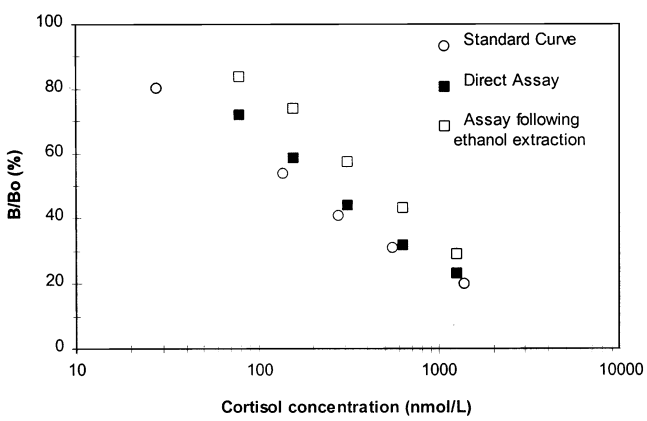

Fig. 1. Dose-response curves obtained using kit standards and known concentrations of cortisol in stripped plasma directly and following ethanol extraction.

Table 1
Percent recovery of exogenous cortisol in charcoal stripped turbot plasma, assayed directly and following ethanol extraction[a]

Sample number	Concentration of cortisol added (nmol l^{-1})	Cortisol recovery before ethanol extraction (%)	Cortisol recovery following ethanol extraction (%)
1	1253.91	90.66	59.74
2	626.95	82.60	47.53
3	313.48	79.71	44.64
4	156.74	76.89	35.93
5	78.37	67.94	42.28

[a] Each sample was processed in duplicate.

read. Using this method, the accuracy of the assay, calculated as the average recovery of exogenous cortisol from spiked samples was 93.4% (Table 2). Validation of parallelism was determined as the similarity between the dilution curves. Serially diluted stripped plasma spiked with known concentrations of cortisol were parallel to the standard curve (Fig. 2). There was no significant difference between the slopes of the two dilution curves derived ($t = 0.388$, d.f. $= 6$, $P > 0.05$).

The precision of the assay, defined as the intra-assay CV for repeated measures of the same samples was 11.7% for a sample containing 346 nmol l^{-1} ($n = 18$), and 7.8% for samples containing 130 nmol l^{-1} ($n = 8$). Reproducibility of the assay, defined as the inter-assay CV for repeated measures of the same samples was less than 11.5% for samples containing 346 nmol l^{-1}, and less than 7.3% for samples containing 130 nmol l^{-1}. The sensitivity of the assay is defined as the least amount of hormone that can be distinguished from zero and calculated as the mean $\pm$ 2.5 S.D. of the blank values. The sensitivity of this assay, using 50 µl of sample per kit tube was calculated as 0.858 ± 2.465 nmol l^{-1} or 3.323 nmol l^{-1}.

In this study average resting turbot plasma cortisol concentrations were 10.13($\pm$3.13) nmol l^{-1} ($n = 5$), and average stressed turbot plasma cortisol concentrations were 162.08($\pm$3.13) nmol l^{-1} ($n = 6$) (equivalent to 3.67($\pm$1.14) and 58.75($\pm$27.15) ng ml^{-1}). There was a highly significant difference between plasma cortisol levels in stressed and unstressed turbot juveniles ($t = 4.964$, d.f. $= 5$, $P < 0.01$).

Table 2
Average recovery of exogenous cortisol (mean $\pm$ S.D.) from spiked samples analysed in duplicate using turbot specific standards

Cortisol concentration added (nmol l^{-1})	Expected value (nmol l^{-1})	Observed value (nmol l^{-1})
0.00	–	25.55(1.62)
15.67	41.22	36.12(4.73)
31.35	56.90	66.83(8.16)
62.70	88.24	81.87(16.49)
125.391	150.94	114.10(8.71)

4. Discussion

4.1. Performance of the assay

The low recovery of exogenous cortisol from spiked samples in this study confirms the incompatibility of using human plasma based standards with turbot blood samples. The improved performance of this method using charcoal stripped turbot plasma spiked with known cortisol concentrations confirms the presence of a matrix effect in turbot plasma [20]. Furthermore, the increase in percent cortisol recovered with increasing cortisol concentrations suggests a differential for interference in the assay with different quantities of cortisol. Due to the excessive variability obtained following ethanol extraction, possibly as a result of the extraction of cortisol with the hexane, this method was disregarded as a tool to improve recovery of cortisol using this RIA technique.

An alternative method to overcome this problem involves the preparation of standards for use with this kit directly in turbot plasma. Using this method the accuracy, measured as the percent recovery of exogenous cortisol from spiked samples, improved to 93.4%. This is comparable to an accuracy of 91% achieved with rainbow trout, *Oncorhynchus mykiss*, plasma using a similar RIA method [4].

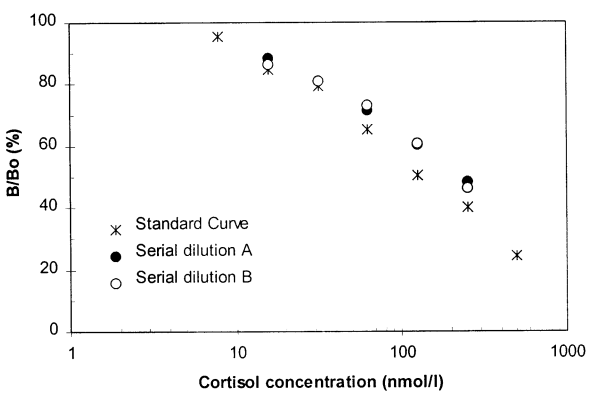

Fig. 2. Curves obtained using standards prepared in stripped turbot plasma, and serial dilutions of spiked plasma samples.

Using 50 μl of each sample per kit tube resulted in a sensitivity of 3.32 nmol l^{-1}. This is comparable to the sensitivity of the unmodified kit which is quoted at 5.5 nmol l^{-1} using 25 μl of each test sample per kit tube. Due to the low levels of cortisol in unstressed turbot plasma, this method renders this kit more suitable for use with turbot, than using the manufacturers recommended 25 μl.

4.2. Cortisol levels in turbot plasma

The range of turbot, *S. maximus* (Rafinesque) plasma cortisol concentrations found in this study, including resting (10.13 ± 3.13 nmol l^{-1}) and stressed (162.08 ± 3.13 nmol l^{-1}) samples was comparable to those found by Waring [20]. Maximum cortisol levels recorded for turbot were considerably lower than those reported for a number of other cultured fish species. Strange [17] reports maximum cortisol levels in stressed Chinook salmon, *Oncorhynchus tshawytscha*, of over 200 ng ml^{-1}. Stressed trout, *O. mykiss*, show similar maximum cortisol levels to those reported for salmon [9]. Peak cortisol levels in both of these species were reported one h after the fish were subjected to a stressor, as is the situation reported for turbot [20]. In this study stressed samples were taken from turbot 60 min after being stressed in an effort to estimate peak cortisol levels. The results suggest that in using RIA kits to measure plasma cortisol levels as an index of stress in turbot, calibrators ranging from almost zero to 100 ng ml^{-1}, or zero to approximately 300 nmol l^{-1} are sufficient.

The quantitative response to different stressors varies for fish species, and so when using cortisol levels to investigate the stress response, the stressor must be standardised. Chemical and physical stressors induce cortisol responses of different magnitudes [15]. A similar situation is seen when different handling stressors are employed [5]. Once these factors are taken in to consideration, RIA kits, using specific standards, based on estimations of accuracy, precision and reproducibility as analysed here, provide a valuable tool for the quantification of stress responses in turbot.

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