

## **REVIEW PAPER**

### **ISSUES IN FISH CORTISOL MEASUREMENT**

**Alexandru-Ioan Lataretu**

*Faculty of Veterinary Medicine Bucharest, 105<sup>th</sup> Splaiul Independentei, District 5, Bucharest, alex\_lata@yahoo.com*

#### **Abstract**

*This paper represents a review of the stress response in fish with emphasis on cortisol, different methods of sampling, advantages and disadvantages. Cortisol is one of the most common measured stress indicator hormone in fish. The reasons why this hormone is often addressed by researchers are: Cortisol can be rapidly and accurately measured using ELISA (enzyme-linked immunosorbent assay) and RIA (radioimmunoassay), the samples can be obtained through a wide variety of harvesting procedures that minimizes stress response in fish (including anesthesia), the plasma cortisol level has a tendency to rise when the fish is exposed to different stressors.*

**Key words:** cortisol, fish, stress response

#### **INTRODUCTION**

The stress response in fish

According to Branson E. J. (2008), in comparison with the stress research in domestic animals, the study of stress in fish has a relatively short history starting with the late 1960s and early 1970s.

Primary response: The hypothalamo-pituitary-interrenal axis

Neil Martin Ruane (2002) mentions in his thesis that fish response to stress is controlled by the hypothalamo-pituitary-interrenal axis (HPI). He also relates that after the stressor is detected, sensory neurons in the brain are activated and direct the information to the hypothalamus. According to van Enkevort et al. (2000) and van den Burg (2002) the main factors that trigger the release of the pituitary peptides are the corticotropin releasing hormone (CRH) and the thyrotropin releasing hormone (TRH), freed by the hypothalamus. The adrenocorticotrophic hormone (ACTH), the  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) and the endorphin are the main peptide hormones produced by the pituitary gland. Although the role of the ACTH hormone to stimulate the production and release of cortisol from the interrenal cells (24) is well known, the function of the  $\alpha$ -MSH hormone and endorphin is still subject to discussion (3; 4; 28). The precursor of cortisol is represented by cholesterol. This sterol is transformed to pregnolone by the

action of the enzyme P450 (18). Afterwards pregnolone is further converted into 11-deoxycortisol by the action of steroidogenic enzymes and finally converted to cortisol by 11 $\beta$ -hydroxylase (18, 10)

*The interrenal gland:*

According to Ruane N.M. (2002), the teleost kidney is composed of a head and body kidney. The head kidney is represented by a wide haematopoietic, endocrine and lymphatic tissue, while the body kidney is composed of nephrons and interstitial lymphoid tissue (27). Milano et al. (1997) describes the anatomy of the head kidney as consisting of the interrenal gland (the correspondent of the adrenal cortex in mammals) and the chromaffin cells (adrenal medulla); these structures are presented as surrounding the postcardinal vein and its collaterals. According to Wendelaar Bonga (1997), the main steroid hormone synthesized by the interrenal cells is represented by cortisol, whereas the chromaffin cells secrete catecholamines. The interrenal gland is represented in *Cyprinus carpio* by clearly defined glandular masses, surrounding the postcardinal vein with branches that infiltrate the head kidney (24). Imagawa et al. (1995) describes the chromaffin cells as being located singly or in clusters in the postcardinal vein walls and delimited by the interrenal cells. The regulation of the interrenal and chromaffin cells by the endocrine system through the circulatory system, is enhanced by their location near the postcardinal vein (24).

Shortly, during the activation of the HPI axis, the corticotropin releasing factor (CRF) induces the pituitary corticotropes to secrete ACTH (2). Some authors (1, 14) suggest that a specific binding protein for CRF (CRF – BP), as well produced in the POA, may have a role in the CRF – mediated ACTH regulation during stress response. Bernier et al. (2009) report that blood-born ACTH in his turn stimulates synthesis and secretion of cortisol into the circulation.

*The secondary response:*

According to Ruane N. M. (2002), the secondary stress response appears consecutively to the neuroendocrine changes that follow the primary stress response; thus metabolism and immune capacity are being influenced. The same author states that in order for an animal to respond to a stressor, a supplementary metabolic cost is required, the organism needing to dispatch energy substrates such as glucose and free fatty acids; the increase in plasma glucose concentration is due to elevated glycogenolysis (breakdown of liver glycogen) and gluconeogenesis (production of glucose), whereas

lipolysis (lipid breakdown) leads to the formation of free fatty acids from triglycerides in fat stores.

The tertiary response:

*The tertiary response* is represented by the adverse consequences of exposure to stressors (5). Branson E. J. (2008) states that the indicators of a tertiary stress response can be: reduction or even ceasing of growth, low body condition score, increased incidence of infection (bacterial, viral, fungal, parasitic), low reproductive status.

**Cortisol measurement:** in blood, bile, whole body homogenates or water.

Cortisol in blood

Edward J. Branson (2008) states that from a research point of view, the blood contains the most relevant concentration of cortisol. Also he recommends sampling the blood with a hypodermic needle and syringe from a lightly sedated fish. The same author mentions that the blood samples can be obtained by heart puncture, from the caudal vein or artery or from the cuvierian ducts (posterior cardinal veins). The problem of this approach, from the author's point of view, is that the time elapsed between the initial disturbance (catching the fish in nets) and blood harvesting should be less than five minutes. If this is not possible, the initial disturbance associated with the sampling maneuver will generate a stress response or possibly increase an already existing response (21). Authors like Marcel Martínez – Porchas et al. (2009) state the following plamatic cortisol values from the literature (table 1):

Table 1. Plasma cortisol values in carp and trout, before and after different stressors (18).

Species	Stressor	Cortisol nmoli/l		Exposure	References
		Prestress	Poststress		
Rainbow trout <i>Oncorhynchus mykiss</i>	Chemical exposure	49	110	Chronic	Benguira et al. 2002
Rainbow trout (diploid) <i>Oncorhynchus mykiss</i>	Handling and confinement	77	698	Acute	Benfey & Biron 2000
Rainbow trout male <i>Oncorhynchus mykiss</i>	Trapping	16	380	Acute	Clements et al 2002
Common carp <i>Cyprinus carpio</i>	Density	19	206	Acute	Ruane et al 2002

An other study by Mackenzie Macintyre C. (2008) states the following results:

Materials and methods: the author describes that samples were harvested from 3699 trouts removed from the pool by netting, immediately transferred into water with anesthetic (2-phenoxy ethanol, Sigma, Dorset, UK) and stunned. Immediately following death, blood samples were harvested using syringes and a heparinised 23 gauge hypodermic needle from the caudal vena cava. The samples were stored on crushed ice. The plasma cortisol values were determined using the radioimmunoassay method as described by Ellis et al. (2004), adapted by North et al. (2006a). Concentrations were reported in ng/ml.

Results: the author reports the following results: average concentration  $\pm$  standard deviation of  $8.29 \pm 13.36$  ng/ml, with differences between system type. The highest cortisol concentrations ( $15.4 \pm 19.62$  ng/ml) were reported in fish provided from cage systems in comparison with raceways where the concentration was  $5.89 \pm 7.81$  ng/ml. For fish from ponds and tanks, the mean  $\pm$  standard deviation for cortisol concentrations were  $7.51 \pm 12.34$  and  $8.25 \pm 14.31$  ng/ml.

Cortisol in the bile:

Branson E.J. (2008) states that cortisol is inactivated and cleared from the body through hepatic biotransformation processes. A study by Pottinger et al. (1992) on rainbow trout, *Oncorhynchus mykiss*, showed that the levels of cortisol metabolites and their conjugates in the bile are significantly higher in fish exposed to a chronic stressor. Thus it is concluded by the author that the analysis of biliary steroid content may provide a useful tool for

identifying stressed fish under conditions where an supplemental sampling stress is inevitable.

Materials and methods: the accumulation of corticosteroids and their metabolites in the bile was measured using radioimmunoassay (RIA) and gas chromatography – mass spectrometry (GC-MS). One of the experiments was represented by: „The effect of acute and chronic stress on plasma cortisol levels, respectively free and conjugated biliary steroid levels in rainbow trout *Oncorhynchus mykiss*”. The author describes that the fish were held in 1500 L capacity circular outdoor tanks with a constant intake of lake water, (30 l/min, with water temperature of 6.5 °C). The fish received daily food except during the experiment. Three groups of 10 fish were netted from their holding tank and sampled after being subjected to 24 h of confinement (first group – chronic stress), 1 h of confinement (second group – acute stress) and immediately in the third group (control). The blood samples were harvested from the caudal region using a heparinized syringe and the bile samples from the gall bladder with a syringe and a needle. The following were determined: cortisol levels, biliary free and total steroid levels

Results: the unstressed fish had a low plasma cortisol concentration  $1.8 \pm 0.3$  ng/ml, in comparison with the 1 h acutely stressed fish ( $83.1 \pm 17.0$  ng/ml), and the 24 h chronically stressed fish ( $129.1 \pm 13.5$  ng/ml). The acutely stressed fish had the following unconjugated steroid levels in the bile:  $53.3 \pm 20.7$  ng/ml not significantly higher in comparison with the control fish ( $51.5 \pm 18.5$  ng/ml) but much smaller than the chronically stressed fish ( $169.1 \pm 24.4$  ng/ml). In the same manner, total (free + conjugated) steroid levels in acutely stressed fish had no major differences

when compared to control fish ( $4.6 \pm 1.2 \mu\text{g/ml}$  in comparison with  $3.4 \pm 1.0 \mu\text{g/ml}$ ) but were significantly higher in the chronically stressed fish ( $13.4 \pm 1.0 \mu\text{g/ml}$ ).

Cortisol in whole body homogenates:

When the fish is too small to obtain a sufficient blood sample, cortisol can be measured in whole body homogenates. It is necessary to humanely kill the fish before homogenising it in a special apparatus (9). In his article, Pottinger et al. (2002) shows that this method is useful in monitoring stress response in small fish:

Materials and methods: Pottinger et al. (2002) used in his study three-spined stickleback that were maintained 4 months until the start of the experiment in outdoor 1000 l flow-through tanks supplied with lake water (10 l/min and ambient temperature  $4-17^\circ\text{C}$  – seasonal range) and light period. Commercial trout fry feed was given to the fish. Two of the experiments conducted were:

Experiment 1: “Effects of an acute stressor”

At time 0, eight fish were taken from an outdoor holding tank (controls). The author relates that, immediately after this, 160 fish sampled from a second holding tank were transferred to eight 2.0 l aerated beakers filled with 500 ml of lake water (20 fish per beaker) and at 0.5, 1, 1.5, 2, 3, 4, 6 and 24 h removed one fish from each beaker (sample size of eight at each time point representing the stressed group). Also at 1, 2, 4, 6 and 24 h after the first sample, groups of eight fish were sampled from the holding tank implying minimum stress (control group).

Experiment 2: “Effects of a chronic stressor with food withdrawal”

At time 0, eight fish were taken from an outdoor holding tank (controls). The author relates that, immediately after this, 105 fish sampled from a second holding tank were transferred to seven aerated beakers filled with 1500 ml of lake water (15 fish per beaker). After 1, 2, 4, 6, 8 and 10 days from transfer, eight fish were sampled from a single beaker (sample size of eight at each time point representing the stressed group). Also after 4, 6, 8 and 10 days from the first sample, groups of eight fish were sampled from the holding tank, implying minimum stress (control group). In both experiments the fish were humanely killed and stored at  $-70^\circ\text{C}$  until

required. The immunoreactivity of cortisol was conducted in ethyl acetate extracts of whole body homogenates by radioimmunoassay (23).

## RESULTS

The control group had the mean whole body levels of immunoreactive corticosteroids in the interval 2-8 ng/g. The acutely stressed fish had a high level of corticosteroids in the first 30 minutes of stress (reaching 35-40 ng/g within 1 h) and remaining significantly elevated in comparison with the control fish, during the entire acute trial (24 h). The mean corticosteroid value of the chronically stressed fish was similar to that of the acutely stressed fish (35 ng/g in the first 24h, respectively 50 ng/g within 4 days). Values decreased later in the study but remained significantly elevated in comparison with the control fish. Food restriction also increased the corticosteroid whole body levels (9.9, 14.1 ng/g in the fasted fish in comparison with 5.5, 8.1 ng/g in the fed fish).

Cortisol in water:

Branson E.J. states that a major part of the circulating cortisol is removed by bioconversion and excretion through the bile. Also according to other authors (30, 13), a major amount of free steroids are passively eliminated from the blood as it passes through the gills. Although this method can reveal important information about the endocrine status of the fish population, in a non-invasive manner (26, 12, 16), there are two major disadvantages of this method (9): first, an enclosed volume of water is required with known inputs and outputs, and second, individual variation is lost.

Ruane N. M. si Komen H. (2003) measured the cortisol in water in order to measure the stress in common carp (*Cyprinus carpio*), caused by increased loading density.

Materials and methods: the authors describe that on day 0 of the experiment fish were organised into two density groups composed of four low-density tanks with 25 fish and three high-density (HD) tanks with 100 fish. Both blood and water samples were harvested from the LD and HD tanks on days 1, 3, 8, 14 and 28. Plasma cortisol concentrations were measured by radioimmunoassay (RIA) and cortisol in the water was measured as described by Scott & Sorensen (1994) for free steroids and according to the instructions of the cartridge manufacturers for glucocorticoid measurements (31)

Results: in time, the plasma cortisol concentration was significantly higher in all the groups in comparison with the first day. The highest value of the plasmatic cortisol due to the increased loading was recorded in day 3. In the HD groups cortisol concentrations were significantly elevated during the experiment, excepting the 14th day. In comparison with the cortisol concentrations in day 1, the LD group had a higher cortisol value in day 8 and the HD group in day 8 and 28.

## CONCLUSIONS

Cortisol appears to be an accurate and accessible stress witness in fish. A major advantage is that it can be determined through a wide variety of methods. Thus, it is concluded that cortisol corroborated with other fish health parameters can be of a real useful tool in evaluating the welfare status of fish in different aquaculture systems.

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