Full Length Article

Effect of Confinement on Water-borne and Whole Body Cortisol in Wild and Captive-reared Rainbowfish (*Melanoteania duboulayi*)

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Abstract

Whole body and water-borne cortisol levels were measured in captive reared and wild Rainbowfish (*Melanoteania duboulayi* Castelnau 1878) subjected to social isolation by confining them in a beaker for 30 min to induce an acute stress response. Wild fish had higher levels of cortisol before and after exposure to a mild stressor and also showed the greatest stress response. The differences in stress responses are likely the result of artificial selection in the captive environment. Importantly, there was a strong linear relationship between whole body and water-borne cortisol in wild and captive reared populations (r^2 =0. 95 and 0.84, respectively) suggesting that non- invasive assays for cortisol provide a valuable alternative for whole body cortisol levels in small fishes. © 2014 Friends Science Publishers

Keywords: Stress response; Wild fish; Hatchery-reared fish; Whole-body cortisol; Water-borne cortisol; Confinement

Introduction

There is growing evidence that hatchery reared and captive bred fish often differ behaviorally, morphologically and physiologically from their wild counterparts (Huntingford, 2004; Zuberi *et al*., 2011). Behavioural traits are considered to be the first traits affected by domestication (Price, 1999) and are often the precursor to alter the physiological stress response in many fish species (Davis, 2006; Woodward and Strange, 1987). Such differences between wild and captive reared fish become a concern when hatchery-reared fish are released into the natural environment for stock-enhancement purposes or when laboratory or hatchery reared fish are used as experimental subjects and results extended to field conditions.

The physiological stress response is common to all vertebrates and can be initiated by many types of environmental changes. The stress response can be characterized by physiological changes, such as elevated heart and ventilation rate and can be detected by examining changes in the blood chemistry parameters including plasma cortisol, glucose, lactate and electrolyte concentrations (Khaliq *et al*., 2013). A number of studies have been conducted where cortisol, the classic stress hormone, was used as a quantitative primary physiological stress indicator in fish (Barton, 2000; Fridell *et al.*, 2007) and has been included in recent studies related to welfare of fish (North *et al*., 2006; Varsamos *et al*., 2006). Although variation in stress-induced cortisol levels is considered as a heritable component (Fevolden *et al*., 1999; Pottinger and Carrick, 1999) but changes in stress responses can also be induced over an individual's lifetime as a result of experience

(Brown *et al*., 2005). Thus the reduced stress response frequently observed in many captive or hatchery reared fish e.g., greenback flounder (*Rhombosolea tapirina;* Barnett and Pankhurst, 1998), winter flounder (*P. americanus;* Breves and Specker, 2005), rainbow trout (*Oncorhynchus mykiss*; Woodward and Strange, 1987), rainbowfish (*Melanoteania duboulayi*; Zuberi *et al.,* 2011) are the result of both long term selective processes operating in the hatchery over many generations and the contemporary rearing conditions of particular individuals.

Cortisol is the principal glucocorticod, generally recognized as a stress marker in fish. It is secreted by the activation of the hypothalamus-pituitary- renal axis (HPI). Under stress conditions, the hypothalamus releases corticotropin-releasing factor (CRF) toward blood circulation and stimulates the secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland which activates cortisol release by the interrenal tissue. Cortisol in turn activates the glycogenolysis and gluconeogenesis and provides enough energy to cope with stressful conditions. In vertebrate including fish, liver acts as a major organ for the cortisol clearance. There, reduction, oxidation and hydroxylation of cortisol occur and the end products of these reactions become water soluble by conjugation with sulfate or glucuronic acid and are excreted in urine (Wilson *et al*., 1998). Like mammals, although the highest proportion of cortisol is eliminated through hepatic processes but bronchial routes also play significant role in the steroid elimination in fish (Ellis *et al*., 2004). According to Scott and Ellis (2007), free cortisol moves across the anterior gill region into the water through passive movement i.e., from higher concentration in plasma to lower concentration in water.

Measurement of concentration in blood is considered as the most common method of assessing the cortisol status of fish (Barton *et al.*, 2005; Quigley and Hinch, 2006). However, many investigators have also used whole-body cortisol levels for assessing the stress response of small fish because of insufficient volumes of blood for the measurements of circulating hormone levels (Pottinger *et al.*, 2002; Sink *et al.*, 2007). To overcome the problems associated with taking blood samples or killing small fish for sampling hormones (e.g. handling stress), non-invasive methods to measure the corticosteroid concentration in fish have been developed (Scott and Ellis, 2007). This new methodology has, for example, been employed to measure the water-borne cortisol levels from large enclosures containing groups of rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) (Ruane and Komen, 2003; Ellis *et al.,* 2004). Water-borne hormones collection techniques have become increasingly popular among behavior ecologists, because they avoid many of the problems inherent in blood sampling (Ellis *et al.*, 2007; Fanouraki *et al.*, 2008; Zuberi *et al.*, 2011). It is vital however, that sufficient knowledge about the relationship between water-borne hormone levels and those circulating in the fish is gained for this to make it a valid and widely accepted technique. While Ellis *et al.* (2004) reported a significant concordance between blood plasma and water-borne cortisol concentrations in the rainbow trout, no one has examined the relationship between whole-body and water-borne concentration, which is the most appropriate technique for small-bodied fishes.

The crimson spotted rainbowfish, (*M. duboulayi*), is a small bodied freshwater fish that is popular in the aquarium hobby due to considerable color variations within the genus (Rodgers *et al.*, 2010). The genus has a widespread distribution and is commonly found in all freshwater habitats in Australia and Papua New Guinea. Despite its extensive use as an aquarium species and laboratory research organism (McGuigan *et al.*, 2003), little is known about its physiological stress response. Its small size and the ease to which it adapts to captivity make them an ideal model organism to study stress response in captive reared and wild populations.

The objective of this study were three; (a) to determine the basal levels of whole body and water- borne cortisol using a flow-through system in wild and captive reared fish, (b) to investigate the effects of confinement stress on cortisol levels in both populations and (c) to determine relationship between whole body and water-borne cortisol in both populations.

Materials and Methods

Experimental Fish

Adult rainbowfish, bred and reared for about fifteen generation in captivity (Kydd and Brown, 2009 for details) were used as a captive reared fish and wild fish were collected from the Orara River, Australia and transported live to Macquarie University. Initially, all fish were housed in glass aquaria (90 × 40 × 40 cm) containing artificial plants and river gravel. Photoperiod was maintained at 12:12 light/ dark using overhead fluorescent tubing. Over the first few days, wild fish were weaned on to commercial flake food (Tetramin) and held in captive conditions for a month prior to experimentation to let them to adjust their life in captivity. This is essential so as to avoid potential confounds during experimentation associated with adjustments to novel surrounds.

Fifteen days prior to experimentation, the test fish were shifted in glass aquaria (40 × 25 × 25 cm) at a stocking density of 5.0 g L^{-1}. These experimental aquaria lacked river gravel (substrate) and were equipped with a heater and a small filter after removal of the sponge for constant temperature of 22.5°C and DO level near saturation (~7.5 mg L^{-1}). The constant rate of flow of 10 ± 0.05 mL min^{-1} of each aquarium was adjusted by connecting them via tubing and a regulator to a reservoir containing aged dechorinated water. The reservoir was set above the test aquaria and equipped with a water heater set to 22.5°C. Outflow of water from each aquarium was also controlled by use of tubing and a regulator. During the experimental period, pH ranged from 6.2 to 6.5, while ammonia was less than 0.25 ppm, and salinity was 0.0 ppm. Fish were provided tetramin flake food once daily between 08:00 and 9:00 AM.

Before investigating the stress response, pre-stress whole body and water-borne cortisol levels were determined by collecting 500 mL water from fish holding aquariums using the flow through system and immediately capturing and euthanizing seven fish from each population with an overdose of buffered MS222 (150 mg L^{-1}). Capturing and killing the fish took less than 30 sec. To remove excess water, individual fish were blotted on paper towel, immediately placed in a zip-lock freezer bag and frozen at −80°C. These data served as time 0 (unstressed samples). The determination of background cortisol levels are essential in order to set the levels of parameters to be used as stress indicators in normally active fish. Studies of stress responses are potentially misleading without such information (Pankhurst and Sharples, 1992). A further seven fish were netted from their aquaria and transferred individually in clean 500 mL glass beaker containing 100 mL of water. After 30 min confinement in the beaker, fish were removed, euthanized and stored at −80°C as described above for cortisol extraction. Water-borne cortisol samples from each beaker were filtered by using Whatman filters to remove particulate matter, followed by filtration through 0.45 μm filter (AcroCap™, GelmanSciences, USA) by using Millipore assembly. After filtration, water samples were either stored at −20°C or extracted immediately following collection. According to Ellis *et al.* (2004), storage of water samples at low temperature does not affect cortisol concentrations.

Hormone Extraction

Water-borne cortisol: The water-borne cortisol extraction procedure was based on the method described by Ellis *et al.* (2004) and Zuberi *et al.* (2011). Briefly cortisol from water samples was extracted by using an activated LiChrolut® reverse phase C_{18} solid phase extraction cartridge (RP-18, 500 mg, 3 mL, 40 - 63 µm, standard PP Merck). The cartridges were fitted to a 20-port vacuum manifold and then primed with 4 ml HPLC grade methanol (CH_3OH) followed by 4 mL ddH_2O. The water samples were then passed into the columns through Tygon® tubing by using the vacuum manifold. Once entire water sample was passed, the cartridges were washed with 2×2 mL ddH_2O and free cortisol was eluted from the columns into 10 mL glass test tube (12×75 mm) by two consecutive 3 mL washes with ethyl acetate. A 6 mL of eluted solvent was evaporated at 45°C using a nitrogen gas stream and the residue was re-dissolved in 500 µL of RIA buffer and kept frozen until assayed.

Whole-body cortisol: Whole-body cortisol was extracted by using a modified method previously used for golden shiners, *Notemigonus crysoleucas* (Sink *et al.*, 2007). Whole frozen individual rainbowfish were thawed, weighed accurately by using electrical balance and placed on a glass petri plate and sliced into small sections. The slices were homogenized using a glass/glass homogenizer with 1mLof phosphate buffered saline (PBS, pH 7.4), disrupted by ultrasonication for 2 min. Probe of sonicater was rinsed into the sample tube with an additional 500 µL aliquot of PBS.

Each sample was extracted thrice with six volume of diethyl ether. For each extraction, 3 mL of diethyl ether was used and then the tubes were vortex for 30 sec and centrifuged at $1000 \times g$ for 15 min at 4°C to separate the ether and aqueous layers. The aqueous homogeneous layer that was at the bottom was frozen at -80°C for 15 min and the top layer containing extracted steroid hormones was decanted into a separate 10 mL glass test tube and evaporated under a gentle stream of nitrogen gas at 45°C. The lipid extract containing cortisol was stored at –20°C until cortisol RIA was performed.

Radioimmunoassay

Free cortisol concentrations were measured using a commercially available RIA kit (competitive binding Coat-A-Count® cortisol kit, Diagnostic Products, California, Inc.). All samples were run in duplicate. The RIA kit was validated for measuring rainbowfish cortisol in water-borne and whole-body extracts by verifying that slopes obtained by serial dilutions of these samples were parallel to the curve created with kit standards (compare slopes, Water-borne cortisol: slope = 0.978, r^2 = 0.995, P = 0.97; whole body cortisol: slope = 0.932, r^2 = 0.998, P = 0.96). To determine the precision of the method the coefficient of variation (% CV) was calculated by comparing the results from the repeated assays and assay of samples at different

time. The intra-assay coefficient of variation for whole body and water-borne cortsiol were 11.3 and 8.47% respectively whereas inter-assay coefficient of variation (% CV) were 3.38% and 5.2% for whole-body and water-borne cortisol respectively.

The extraction efficiency (% recovery) from water sample was evaluated by adding an equal volume of 10, 50 and 100 ng mL^{-1} standards supplied with the RIA kit to water samples collected from main reservoir which supplies dechlorinated water to experimental aquaria. Water samples containing cortisol were then passed through extraction cartridges. The cortisol was eluted from the column using $2\times$ 3 mL volumes of ethyl acetate and cortisol concentration was quantified using RIA as described above. The minimum observed recovery of the methodology (extraction and ethyl acetate elution) was 93.9%.

The extraction efficiency from whole body was assessed by adding redioinert cortisol in charcoal stripped whole body homogenate at predicted concentrations of 10, 20, 50 and 100 ng g^{-1}. Cortisol was extracted with the same procedure as adopted for sample and was quantified with RIA. The extraction efficiency was found greater than 95%.

All cortisol values are reported as ng g^{-1} fish for whole body cortisol, ng L^{-1} for cortisol concentration and ng $g^{-1} h^{-1}$ for cortisol release rate.

Statistical Analysis

Data obtained from the experiment was expressed as mean±SEM. Linear regression was adopted to examine the relationship between whole-body and water-borne cortisol while the difference between wild and captive-reared rainbowfish stress responses before and after confinement stress was analyzed by using ANOVA in SPSS statistical package (Software version 16.0 Chicago, IL).

Results

Captive reared fish showed both reduced baseline levels of cortisol ($F_{1, 24}$ = 105, P < 0.001; $F_{1, 24}$ = 28, P < 0.001 for whole body cortisol concentration and water-borne cortisol release rate, respectively), and a reduced stress response to confinement relative to wild fish using both extraction techniques ($F_{1, 24}$ = 1238, P < 0.001; $F_{1, 24}$ = 302, P < 0.001 for whole body cortisol concentration and water-borne cortisol release rate, respectively). The water-borne cortisol release rate increased from 0.081 to 0.628 ± 0.059 ng $g^{-1} h^{-1}$ in wild fish and from 0.062 ± 0.004 to 0.29 ± 0.05 ng $g^{-1} h^{-1}$ in the captive reared fish (Fig. 1). The whole body cortisol showed a similar pattern. In wild fish the level increased from 4.28 ± 0.21 to 40.43 ± 3.66 ng g^{-1} whereas in the captive reared population it increased from 1.57 ± 0.2 to 25.75 ± 2.26 ng g^{-1} fish (Fig. 1). In both instances there was a significant population by treatment interaction indicating that the rate of increase from the baseline was greater in the wild population ($F_{1, 24}$ = 16, P < 0.001; $F_{1, 24}$ = 6.8, P < 0.001 for whole body and water-borne cortisol concentration,

respectively; Fig. 1). Quantitatively and qualitatively similar results were obtained using both approaches.

Relationship between Whole Body and Water-borne Cortisol

A highly significant positive relationship between whole body and water-borne cortisol in the wild population (r^2=0.95, y = 0.0145x – 0.0301, P < 0.001) and the captive-reared population (r^2 = 0.84, y = 0.0087x – 0.0587, P < 0.001) (Fig. 2 and 3) was observed.

Discussion

This study clearly demonstrated that captive-reared and wild crimson spotted rainbowfish have dramatically different stress responses. In terms of their stress hormone concentrations determined by both whole body and water-borne concentrations, the two populations showed different levels of cortisol during the control condition (background levels) and under a mild stress condition induced by social isolation. Moreover, the change in cortisol concentration induced by social isolation was greatest in the wild fish. Captive-reared fish showed attenuated stress responses, which is likely a function of prolonged artificial selection in the captive environment. In addition, we report very strong correlations between whole body cortisol concentrations and water-borne cortisol for both the populations. This latter finding suggests the use of an indirect measure of cortisol circulating in the surrounding water, which is a suitable replacement for using whole body extractions that involve sacrificing small-bodied fish. Use of water-borne cortisol measurements in stress studies is also ideal in avoiding false increases in the cortisol due to handling or anesthetics used prior to animal sacrifice.

Although in wild fish, the basal levels of cortisol were significantly higher as compared to captive reared fish, the absolute difference between them was rather small and comparable to the range reported in other small-bodied species. The background (non-stressed) mean whole body cortisol levels of captive reared and wild rainbow fish was 1.57 and 4.28 ng g^{-1} fish respectively which is in agreement of cortisol values among adult *Gasterosteus aculeatus* held under control conditions (2-8 ng g^{-1} fish; Pottinger *et al.,* 2002). Similarly mean resting whole body cortisol levels in zebrafish (*Danio rerio*) range between 1-3.2 ng g^{-1} fish (Ramsay *et al.,* 2006, 2009). In addition, these values are comparable to those previously reported in this species behaving normally in a small aquarium (Zuberi *et al.,* 2011).

Social isolation by confinement to a glass beaker resulted in a considerable elevation of whole-body cortisol levels in both wild (40.43±3.66 ng g^{-1} fish) and captive reared (25.75±2.26) rainbowfish despite the fact that this is considered a mild stressor for this facultative schooling species (Brown *et al.,* 2005). Wild fish probably perceive capture and social isolation as very threatening,
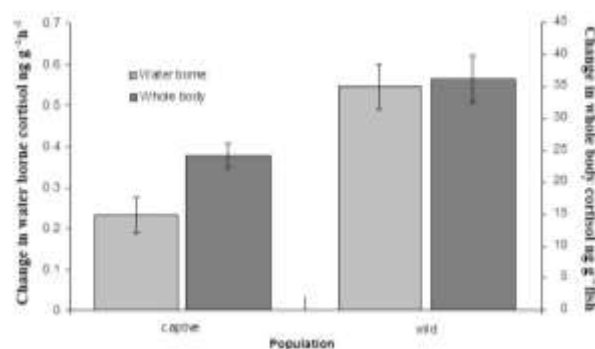

Fig. 1: Change in whole body and water-borne cortisol after 30 min beaker confinement in wild and captive reared fish. Data are represented as Mean±SE. Means followed by the different letter are significantly different (P < 0.05). (ANOVA followed by Tuckey test)
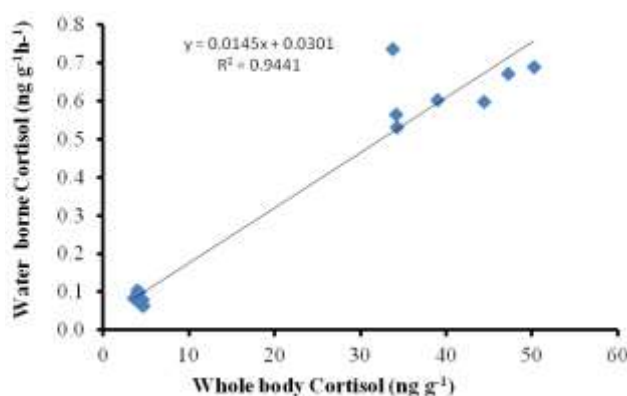

Fig. 2: Significant positive relation between whole body (ng g^{-1} fish body) and water borne (ngg^{-1}h^{-1}) cortisol in wild rainbowfish populations

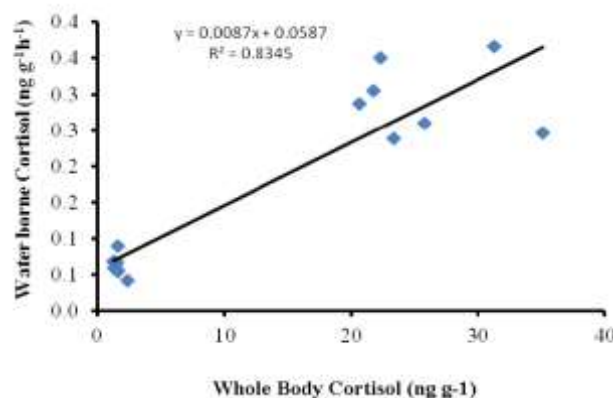**Fig. 3**: Significant positive linear relation between whole body (ng g^{-1} fish body) and water borne (ng g^{-1}h^{-1}) cortisol in captive reared rainbowfish populations

whereas captive fish are accustomed to multiple sources of disturbances from their life in the laboratory. We have

previously shown, for example, that the captive population has reduced schooling behavior (Kydd and Brown, 2009) and thus social isolation is not likely to impose a significant stressor on this population. The strength of the stressor clearly changes the stress response in fish species. Whole body cortisol of mildly stressed zebrafish (*Danio rerio*) was only 9.0 ng g^{-1} fish (Ramsay *et al.*, 2006). In three spined sticklebacks, the whole body cortisol levels in response to chronic crowding (confinement of 15 fish for 5 days in 1500 mL water) were 35 ng g^{-1} fish (Pottinger *et al.*, 2002). Whole-body cortisol levels of golden shiners that had been exposed to multiple stressors (capture, transportation and grading) within a 24 h period ranged between 46.6 – 65.6 ng g^{-1} fish (Sink *et al.*, 2007). Similarly, rainbowfish that was attacked by a simulated predator showed cortisol release rates fourfold greater than those reported here, although the peak response was significantly delayed in the captive population (Zuberi *et al.*, 2011). Rearing environment, strength of stressful stimuli, life stage as well as individual/species difference in the cortisol response to confinement may explain differences between the results reported by investigators (Barton, 2002). Despite the species/individual differences in cortisol response to a stressor, however, most studies to date have shown a 4-16 fold increase in cortisol after the application of a mild or more severe stressor.

Reduced stress response as a result of domestication has been widely reported in a range of vertebrate species including fish, mammals and birds (Woodward and Strange, 1987; Salonius and Iwama, 1993; Künzl and Sachser, 1999). A reduction in the physiological stress response is likely due to the artificial culture environment that relaxes the natural selective pressures operating in the wild (e.g. predation) and imposes new selective agents that cause the population to adapt to life in confinement. Such behavioral and physiological changes as a result of life in captivity can occur very rapidly, in some instances in just a few generations (Huntingford, 2004). It is likely that most individuals that are not suited to life in captivity (high crowding, noisy and constant handling etc) soon die and only those that can cope with the high stress environment survive and are deliberately (or incidentally) selected as breeders to contribute to the next captive generation. Thus it is likely that many hatcheries unintentionally alter the behavior and physiology of the animals they are rearing. If the fish are intended to stay in captivity (e.g. aquaculture for food or the aquarium hobby) then such selection is likely to be beneficial. However, if the fish are released into the wild to bolster natural populations, then clearly the effect is going to be detrimental, especially if the released fish interbreed with wild stocks.

There was variation in individual whole body cortisol levels in response to confinement in both population but the level of variability was considerably higher in wild population in comparison with the captive-reared fish. This reduced variability in stress response at the population level is not uncommon in other fish studies (Wendelaar Bonga,

1997; Barton, 2002). Whole-body cortisol levels in chum salmon (*O. keta*) 2.5–20 ng g^{-1} (de Jesus and Hirano, 1992) and in developing Japanese flounder, *Paralichthys olivaceus,* 2.5–11 ng g^{-1} fish (de Jesus *et al.*, 1991) and silver carp, *Hypophthalmichthys molitrix,* 9.8 - 38.26 ng g^{-1} fish (Kausar *et al.*, 2013) suggest that significant individual variation exists within species and may be indicative of behavioral type or personality (Huntingford *et al.*, 2010; Raoult *et al.*, 2011). According to many investigators, variation in stress-induced cortisol levels has a heritable component (Pottinger and Carrick, 1999) and can be altered through natural and artificial selection (Pottinger and Carrick, 1999; Fevolden *et al.*, 2002). The captive-reared fish that we used in this study were about the fifteenth-generation inbred line and are likely to show reduced genetic variability as a result of repeated selection for low stress phenotypes.

Sampling of blood for hormone assays in small fish is problematic as the blood volume is not enough to provide measurements of circulating hormones. To overcome this problem, whole-body cortisol can be used to evaluate the stress response in small adult fishes (Pottinger *et al.*, 2002; Sink *et al.*, 2007). Both these methods, however, involve sacrificing the animal, that prevent further sampling for hormone levels over time and under variable conditions in the same individuals. Alternative, non-invasive methods have been developed to measure water-borne hormones in single-housed small fish (Earley *et al.*, 2006). These studies follow the validation procedure for water-borne hormone (Ellis *et al.*, 2004; Scott and Ellis, 2007), which have shown that water-borne cortisol concentrations, correspond with blood plasma levels. Wild rainbowfish rarely survives more than a year and rarely exceeds 3 g in body weight, thus whole-body and water-borne cortisol procedures are the most appropriate method for evaluating the stress response in these small fish. Here we found a highly significant positive relationship between whole body and water-borne cortisol in both wild (Fig. 2) and captive-reared (Fig. 3) rainbowfish. The procedure, therefore, provides a good basis for a non-invasive stress assay for small fishes.

In conclusion, higher levels of cortisol before and after exposure to a mild stressor in the wild fish compared to the captive reared fish or differences in stress responses are likely the result of artificial selection in the captive environment. Furthermore, a strong positive linear relation between whole body and water-borne cortisol in both wild and captive reared populations suggested that non-invasive assay provide a valuable alternative for whole body cortisol levels in small fishes.

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