**Is gill cortisol concentration a good acute stress indicator in fish? A study in rainbow trout and zebrafish**

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**Abstract**

Cortisol is the main biomarker of physiological stress in fish. It is usually measured in plasma, which requires blood collection. Though cortisol is produced in the anterior kidney, it can diffuse easily through cell membranes due to its lipophilic nature. Taking advantage of that, some non-invasive techniques have been developed to measure cortisol directly in the water from fish-holding tanks, in skin mucus or in scales. In this study, we explored the possibility to analyze fish cortisol from gill filaments as a reliable acute stress marker. Our results show that gill cortisol levels correlate well with plasma cortisol levels in both rainbow trout and zebrafish exposed or not to an acute stress protocol. Measuring cortisol in gill filaments increases the available possibilities for stress assessment in fish. Although this approach should yet be tested for its use with other stressors, it has several advantages: In relatively large fish (i.e. above 30 g) gill cortisol levels could be measured *in vivo*. Sampling of gill biopsies is very fast and easy, and the procedure does not induce stress if properly performed, making it an ideal option for *in vivo* stress assessment in the field. In small fish, the use of gill tissue to measure cortisol has important technical advantages with respect to the current methods using whole-body homogenates. Gill homogenates could be used directly for ELISA cortisol analysis, avoiding the need of tedious and expensive cortisol extraction protocols, and, since no organic solvent is required, contributing for a more environmentally friendly analysis.

**Keywords**: cortisol; fish; gill; stress; trout; zebrafish

**Introduction**

The corticosteroid cortisol is the most commonly measured stress indicator in fish as it is in humans (Ellis et al., 2012; Mommsen et al., 1999) and, with some limitations, could be also used to assess fish welfare (Ellis et al., 2012; Moberg, 2000; Mormède et al., 2007). Along with the release of catecholamines (noradrenaline, NA, and adrenaline, A) from the chromaffin cells, the release of cortisol into circulation from interrenal cells is part of the primary response to stress in fish. The use of cortisol as an acute stress marker above other candidates has been shown to be advantageous because of different reasons: 1) The release is not so fast to prevent their use as stress marker, like in the case of catecholamines, making easy to obtain pre-stress measurements (Ellis et al., 2012; Mommsen et al., 1999); 2) The cortisol response to stress is intense, providing clear and easy-to-detect differences between stressed and non-stressed animals (Mommsen et al., 1999); 3) The dynamics of the stress response are relatively fast and the observed levels relate well with recent events experienced by the animal (Ellis et al., 2012; Gesto et al., 2013); 4) The intensity of the response correlates with the intensity of the stressor for a given species (Gesto et al., 2015); and 5) Cortisol analysis with immunological techniques is relatively inexpensive, easy and fast (Ellis et al., 2012).

Cortisol is mostly measured in plasma by radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA). In the case of eggs, larvae or small adult fish, cortisol is usually extracted from whole-body homogenates by using organic solvents such as ethyl acetate or diethyl ether, among others (Egan et al., 2009; Sadoul et al., 2015). This technique has been applied to many fish species, from zebrafish to sturgeon (Barcellos et al., 2011; Egan et al., 2009; Pottinger et al., 2002; Ramsay et al., 2006; Sadoul et al., 2015; Sakakura et al., 1998; Sink et al., 2007). Also, techniques to measure cortisol directly in fish-holding water are now available (Ellis et al., 2004; Felix et al., 2013; Scott and Ellis, 2007). Each protocol for cortisol measurement has different advantages and drawbacks, so researchers have to choose one of the available methods depending on their experimental requirements.

Here, we explored the possibility of quantifying cortisol levels in gill filaments as a reliable acute stress marker. This approach can be applied for both relatively large and small fish, with different advantages in each case. In the case of relatively large fish, the technique provides a simple, fast and minimally invasive way to sample the fish, avoiding the need to puncture the blood vessels. In the case of small fish, it provides a more simple way to measure cortisol levels after fish sacrifice than the measurement in whole-body homogenates. Regarding this, gill filaments constitute a much smaller tissue and a cleaner biological matrix than whole fish body, allowing to measure cortisol without the necessity of an extraction procedure, highly decreasing the time, effort and both economic and environmental costs of cortisol measurement.

We therefore performed a series of experiments using rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) as models for relatively large (tens of grams or more) and small (a few grams or less) fish, respectively, to evaluate the reliability of gill cortisol quantitation as a potential stress marker in fish. In trout, we also assessed the potential use of the gill filament biopsies as a minimally invasive technique for assessing stress *in vivo*, by evaluating the stressor potential of sampling gill filaments form the fish.

**Materials and methods**

**Animals**

Rainbow trout (*Oncorhynchus mykiss*) were purchased at a local trout farm (Javier de la Calle, A Estrada, Spain) and transported to the facilities of the University of Vigo. They were maintained in 100 L tanks (20 fish per tank) in a flow-through freshwater system for at least two weeks before performing any trial. The fish were kept at 14 ± 1 °C under a 12L: 12D photoperiodic regime and daily fed (0.5-1.0 % body mass) with commercial dry pellets (Aquasoja, Ovar, Portugal).

Zebrafish (*Danio rerio*) were purchased at ZF Biolabs (Tres Cantos, Madrid, Spain) and maintained in 3 L aquaria (8 fish per tank) in a recirculating water system at 26 ± 1 °C under a 14L: 10D photoperiod. Fish were fed daily twice with commercial pellets and a supplement of *Artemia* spp nauplii was given every other day.

The experiments described comply with the Directives of the European Union Council (2010/63/EU), and of the Spanish Government (RD 55/2013) for the use of animals in research. All protocols were approved by the Ethics Committee of the University of Vigo. Sampling and sacrifice were performed under deep anesthesia minimizing animal suffering.

**Experimental design**

**Experiment 1: Exposure of rainbow trout to acute stress**

Twenty juvenile rainbow trout (72.4 ± 7.8 g body mass) were used in this experiment. A group of ten trout was exposed to an acute stress protocol while another group was not treated and served as control. The stress protocol consisted of a 3-min period of chasing with a net. Thirty minutes after stress, the fish were deeply anesthetized with 2-phenoxyethanol (0.2 %, directly into the tanks) and sampled. Several gill filaments (3 to 5, approx. 10 - 20 mg tissue) were rapidly clipped from the lower region of the left external gill arch (Fig. 1). Caution was taken in order to cut the filaments just above the septum to avoid substantial bleeding (McCormick, 1993). Immediately afterwards, a 0.1 mL blood aliquot was taken by caudal puncture with a heparinized 1 mL syringe and the fish were returned to new, anesthetic-free, tanks. The whole sampling procedure took less than 30 seconds per fish. Plasma samples were obtained by centrifuging blood (6000 x g, 10 min, 4 °C). Both gill filaments and plasma were rapidly frozen on dry ice and stored at - 80 °C until analysis.

**Experiment 2: Evaluation of the stressor potential of gill biopsy in rainbow trout**

In this second experiment we aimed to investigate whether sampling the gill filaments is stressful for the fish. In total, 54 trout (78.3 ± 9.0 g body mass) were used in this experiment. Groups of 6 trout were randomly assigned to nine 30 L experimental tanks, where they were left to acclimate for one week. Three replicate tanks were then randomly assigned to one of the following experimental groups: Control, “gill biopsy” or “blood extraction”. Fish were anesthetized in their tanks by adding 0.2 % 2-phenoxyethanol into the water. Several gill filaments (3-5, from the right external gill arch) or a 0.1 mL blood sample was taken from the fish from the “gill biopsy” or the “blood extraction” experimental groups, respectively, and they were then rapidly transferred to recovery tanks. The sampling took about 10-15 seconds per fish and the samples were discarded (since they were only taken to evaluate sampling as a potential stressor). The fish in the control group were air-exposed for 15 seconds and returned to a recovery tank. One hour after sampling, the fish were anesthetized as described above, and 3-5 filaments from the left external gill arch and a 0.1 mL blood sample were taken from all fish. Plasma samples were obtained by centrifuging blood (6000 x g, 10 min, 4 °C). Both gill filaments and plasma were rapidly stored at - 80 °C.

**Experiment 3: Exposure of zebrafish to acute stress**

A total of 24 adult zebrafish (0.40 ± 0.06 g body mass) were used in this experiment. A group of 12 zebrafish was exposed to an acute stress protocol while another group of 12 fish was not exposed to stress and served as control. The acute stress consisted of netting the fish, putting them in 250 mL beakers (four fish per beaker) and hitting faintly the beaker against a table several times during a 5 min period. After the stress protocol, the fish were returned to their aquaria and were netted again 30 min later for sampling. Control fish were directly netted for sampling. For sampling, fish were anesthetized in MS-222 solution (168 mg L-1) (Westerfield, 2007) buffered with sodium bicarbonate, and then a 4 µL blood aliquot was sampled by caudal puncture. After that, all the gill arches of the left side were sampled and the fish was sacrificed by decapitation. Plasma samples were obtained by centrifuging blood (6000 x g, 10 min, 4 °C). Both gills and plasma samples were rapidly stored at -80 °C.

**Biochemical analyses**

Cortisol levels in plasma and gill filaments were analyzed using a commercial ELISA kit (Cayman Europe, Tallinn, Estonia), following manufacturer’s instructions. Plasma was used directly after dilution (1/300, with ELISA buffer), while gill tissue was first homogenized with an ultrasonic homogenizer in 120 µL of phosphate-buffered saline (PBS, pH=7.33). After centrifugation, the supernatant was diluted (1/5, with ELISA buffer) and used for the ELISA assay. According to manufacturer’s instructions, preliminary tests were performed to assess for the necessity of purifying plasma and gill homogenates before the ELISA assay. Linearity tests were performed with four different dilutions of the samples. The linearity was good (R2= 0.987 for plasma and R2= 0.990 for gill). Calculated cortisol levels for the different dilutions differed less than a 20 %, and therefore, no extraction was required.

In trout, plasma cortisol levels were expressed by mL of plasma. In the case of the gill, cortisol data were normalized by tissue wet weight, tissue protein content, or tissue hemoglobin content. Finally, the normalization per protein content rendered the best results and was therefore used for the graphs. In zebrafish, plasma cortisol data were normalized by plasma protein content since the results were more homogeneous than normalizing by plasma volume. Gill cortisol levels were normalized by gill protein content.

Protein concentration was assayed with the bicinchoninic acid method (Smith et al., 1985). The hemoglobin concentration, as well as plasma glucose and lactate concentrations, were assessed with commercial colorimetric kits from Spinreact (Girona, Spain), following manufacturer’s instructions.

Plasma concentration of catecholamines (NA and A) was quantified by HPLC as previously described (Gesto et al., 2013).

**Statistics**

Student´s t-tests were used to compare cortisol levels between control and stressed rainbow trout or zebrafish. One-way ANOVA followed by Tukey’s tests were used to compare groups in experiment 2. Correlations between plasma and gill cortisol were analyzed by calculating the Pearson product-moment correlation coefficient. Significance level was set at P ≤ 0.05 for all comparisons.

**Results**

In rainbow trout, thirty minutes after the 3-min stress protocol, plasma cortisol levels increased 4 times with respect to control fish (Fig. 2). Similarly, when looking at the gill cortisol levels, they increased 4.9 times with respect to control animals. Both measurements showed a very good correlation (Pearson´s correlation coefficient= 0.944; P=1.5 x 10-33). For those correlations the data from both experiment 1 and experiment 2 were used.

The results of experiment 2 showed no significant differences in any of the stress markers among the different fish groups: controls, gill-sampled fish or blood-sampled fish (Fig. 3), demonstrating that gill biopsies do not induce acute stress in the trout, and therefore, could be considered a mild procedure.

As explained above, plasma protein content was used for normalizing zebrafish plasma cortisol data (Fig. 4). Nevertheless, for comparative purposes, the cortisol levels expressed in ng mL-1 were 17.2 ± 4.2 ng mL-1 and 108.0 ± 30.7 ng mL-1 for control and stressed fish, respectively. The acute stress protocol induced 6.1 and 6.2 fold increases in the fish plasma and gill cortisol content, respectively (Fig. 4). The correlation between plasma and gill cortisol content was also very consistent in this species (Pearson´s correlation coefficient= 0.909; P=6.1 x 10-13).

**Discussion**

Here we evaluated a new approach to assess in fish the levels of a stress marker such as cortisol, which could provide researchers with more flexibility at the time to design their experimental procedures. To date, cortisolemia has been used in fish, as in humans, as the main physiological indicator of stress, due to its important advantages over other potential stress markers. Traditionally, cortisol levels have been measured in plasma, although in the 90’s of the past century, cortisol began to be measured also in whole body homogenates due to the necessity to measure it in eggs, larvae or very small fish species (de Jesus et al., 1991, 1993; Yamano et al., 1991). Nowadays, it is also possible to evaluate cortisol levels with non-invasive techniques such as measuring the levels in fish-holding water, in skin mucus or in scales (Aerts et al., 2015; Bertotto et al., 2010; Ellis et al., 2004; Felix et al., 2013; Scott and Ellis, 2007). All those techniques have specific advantages, but they also have several drawbacks. For example, the procedures for measuring cortisol from whole-body homogenates are mainly restricted to very small animals, and the process is lengthy and technically tedious. Also, when measuring cortisol non-invasively in the water, the main inconvenient is that the information regarding individual variations among the fish in a tank is lost.

Our results show that the measurement of cortisol levels from gill filaments is a reliable indicator of the general cortisolemia in the fish. The correlation between the cortisol levels in plasma and gill is good, in animals exposed or not to stress. For zebrafish, the plasma-gill cortisol correlation was not as good as in trout, probably reflecting the lower number of fish used and also the difficulties associated with the blood collection procedure. Regarding the latter, the tiny volume of blood sample taken could result in a higher chance of contamination of the sample with water or other body fluids. For that reason, we tested two different ways of normalizing plasma cortisol data in zebrafish: by plasma volume (as in the trout) or by plasma protein content. The latter showed better performance and was therefore utilized for graphs and statistical analyses. Similarly, in the case of the gill, we tested three different ways to normalize gill cortisol data in both trout and zebrafish: by gill wet mass, by gill protein content or by gill hemoglobin content. Hemoglobin was used as an indicator of the amount of blood within the gill, since it is known that stress can induce structural alterations in the gill (Wendelaar Bonga, 1997) and that could lead to differences in the gill blood content between control and stressed fish (and that would have been a jeopardizing factor when trying to assess stress from gill cortisol). Although our results showed that the levels of gill hemoglobin were not altered by stress, the normalization by gill protein content rendered better results and therefore, was used for statistical analyses and for the graphs.

Measuring cortisol from gill filaments has several interesting characteristics, but the advantages are different depending on the size of the fish to be sampled. For relatively large fish (i.e. above 30 g), such as trout juveniles, the sampling of gill filaments *in vivo* is fast and simple (clipping some gill filaments requires only a few seconds), and complications are rarely observed, while puncturing the fish for blood extraction could have important welfare consequences if the blood vessels are damaged, particularly when the fish are close to a critical size. In this sense, the technical expertise required for the gill biopsies is lower than that needed for blood collection procedures. Our results showed that, under proper anesthesia, the gill biopsy is a very mild procedure, which evoked no stress response when compared to control fish. Furthermore, we monitored biopsied fish for 3 weeks after sampling and observed no alterations in behavior or health, and no signs of inflammation or infection in the gill. Though, as reported before (McCormick, 1993), gill filaments showed no sign of regeneration. In this regard, other studies had also shown that this kind of gill biopsies have neither short-term nor long-term consequences for the fish (Martinelli-Liedtke et al., 1999; McCormick, 1993).

In view of all that, it seems that the gill biopsy does not compromise fish welfare. In an international context of growing concern about the use of animals for scientific purposes, it is important to minimize the risk of threatening the animal well-being. The safety, simplicity and swiftness of this procedure for stress assessment could be considered as a contribution to the refinement of sampling procedures used in fish, therefore complying with the three R’s principle, which was proposed by Russel and Burch (1959) as guiding principle for a more ethical use of animals, and which are nowadays among the main objectives of the current international regulation regarding the use of animals for scientific purposes (see for instance European Union Directive 2010/63/EU).

Regarding small fish, the advantages of measuring cortisol from gill tissue are different. In small fish, gill sampling, similarly to blood collection procedures, would not allow for quantifying cortisol *in vivo*. This is because the relatively large mass of gill that would be required for cortisol analysis. We used all gill arches from one side of the zebrafish (which constitute less than 10 mg tissue for a 0.6 g zebrafish). In this case the main advantage relays on the simplification of the technical procedures for cortisol analysis. For the assessment of cortisol in small fish, most researchers utilize whole-body homogenates, since blood extraction is difficult to carry out. Cortisol should be extracted prior to the analysis due to the complex biological matrix of those homogenates. Such extractions are time- and money-consuming procedures that require relatively large quantities of organic solvents such as ethyl acetate or diethyl ether, among other reagents (Egan et al., 2009; Ramsay et al., 2006; Sink et al., 2007). On the contrary, our preliminary tests showed that gill homogenates require no extraction procedures before the ELISA assay. Therefore, the measurement of cortisol in 10 mg of gill tissue only requires 100 µL of PBS per sample, previously to the ELISA assay, thus contributing to a simpler, faster and cheaper analysis. Also, the analysis of cortisol from gill tissue is environmentally friendly, since the use of organic solvents is not required, thus supporting the so-called “green chemistry” (Anastas and Eghbali, 2010; Kerton, 2009).

In conclusion, the measurement of cortisol in gill filaments constitutes an innovative, alternative approach to assess acute stress in fish. In the case of small fish, measuring cortisol in the gill is easier, faster and cheaper than the current techniques used for whole-body cortisol analysis. In the case of larger fish, gill biopsies are not stressful, and are technically fast and very simple, resulting in little risk/damage to the fish and making this procedure an ideal option for assessing stress *in vivo*, especially in cases were simplicity could be a key factor, such as when sampling wild fish in the field. Besides plasma cortisol, gill cortisol could be used to measure stress in fish, which may provide researchers with more flexibility at the time to design their experimental procedures. Further research would be required to assess the correlation between gill and plasma cortisol in fish exposed to other kind of stressors or stress durations (i.e. chronic stress).

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**Conflicts of interest: none**

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**Figure captions**

**Fig. 1** Schematic drawing of the left external gill arch of rainbow trout, showing the region were the tips of 3-5 filaments were biopsied for experiments 1 and 2 (see text for more details)

**Fig. 2** a and b: Plasma and gill cortisol levels of rainbow trout after an acute chasing stress protocol. Bar represents the mean ± SEM of n = 10 fish. \*Statistically different from control group (P < 0.001); c: Correlation between plasma and gill cortisol levels in rainbow trout from experiments 1 and 2 (see the text for more detailed information)

**Fig. 3** Cortisol levels in plasma and gill, and plasma levels of catecholamines (noradrenaline, NA, and adrenaline, A), glucose and lactate of rainbow trout, one hour after exposure to gill biopsy (“biopsy”) or blood extraction (“blood”) procedures. Bars represent the mean ± SEM of n = 18 fish. There were no significant alterations in any of the parameters (one-way ANOVA; P = 0.05)

**Fig. 4** a and b: Plasma and gill cortisol levels of zebrafish after an acute chasing stress protocol. Bar represents the mean ± SEM of n = 12 fish. \*Statistically different from control group (P < 0.001); c: Correlation between plasma and gill cortisol levels in zebrafish from experiments 3 (see the text for more detailed information)

**Figure 1**

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**Figure 2**

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**Figure 3**

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**Figure 4**

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