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Cortisol Detection in Fish Scales by Enzyme Immunoassay

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Abstract:	The study of fish stress is usually assessed by measuring blood cortisol. Nevertheless, blood provides only a snapshot of the hormonal profile at one point in time. An alternative source of cortisol may be found in scales, providing a new approach for assessing long-term hormonal levels. The present study aimed to develop and validate a methodology for detecting cortisol in scales of goldfish (Carassius auratus). The study highlights the importance of an initial isopropanol washing procedure to completely eliminate external contaminations of cortisol. Additionally, the biochemical validation of the enzyme immunoassay verifies the ability to detect cortisol with repeatability and reliability in goldfish scales. In conclusion, this study provides validated information about a new methodology to measure cortisol in scales. The incorporation of this biomarker could provide retrospective hormonal measurements from species and time periods that are usually difficult or impossible to obtain, thus offering key data of an animal's physiology.

Cortisol Detection in Fish Scales by Enzyme Immunoassay

Running title: Cortisol detection in fish scales

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Introduction

The study of fish stress responses are usually assessed by quantification of short-term changes in blood cortisol (Baker, Gobush, & Vyne, 2013), the main GC in most teleost fish (Mommsen, Vijayan, & Moon, 1999). Nevertheless, blood provides only a snapshot of the hormonal profile at one point in time. An alternative matrix for cortisol measurement may be found in fish scales. A recent publication showed that the analysis of cortisol in scales could constitute a method for retrospective assessment of fish GC secretion over extended periods of time (Aerts *et al.,* 2015). However, little information concerning the laboratory processing and validation of cortisol measurement in scales is available. Accordingly, the present study was focused on developing and biochemically validating the methodology for detecting cortisol deposited inside the scales of goldfish *Carassius auratus* (L. 1758).

External sources of cortisol, presumably coming from the fish skin mucus (Bertotto *et al.,* 2010), should be previously removed in order to study a more tightly bound fraction incorporated in the interior of the matrix. While isopropanol has been the wash solvent of choice to remove external contaminations of GC in mammal hair (Davenport, Tiefenbacher, Lutz, Novak, & Meyer, 2006; Tallo-Parra, Manteca, Sabes-Alsina, Carbajal & Lopez-Bejar, 2015), in fish, only ultrapure water has been employed (Aerts *et al.,* 2015). Hence, the first experiment explored the appropriateness of water and isopropanol as solvents to decontaminate the scale without compromising the inside hormone contents. We additionally aimed to test the suitability of an enzyme immunoassay (EIA) in the quantification of cortisol concentrations in scales, since the assay procedure

should always be properly validated for each new species and matrix (Buchanan & Goldsmith, 2004).

Materials & Methods

Animals and sampling

Twenty goldfish were euthanized with an overdose of MS-222. Immediately after sacrifice, whole body scales were removed and thoroughly mixed to create two separate pools, one for each experiment.

Washing procedure validation

The pool of scales was uniformly split into two groups given by the type of wash solvent evaluated; water and isopropanol. Each group was subdivided into three different treatment conditions; for one, two or three consecutive washes, with three replicates per treatment. Each replicate was composed by 300 mg of pooled scales introduced into polypropylene tubes. Three millilitres of solvent was added to each tube and vortexed for 2.5 min. Afterwards, the supernatant was separated for further analysis. The process was then repeated once or twice in accordance to the treatment assigned. Once the scales were dried they were minced with a ball mill and 75 mg of each powdered sample were incubated with 1.5 ml of methanol for 18 h. Following extraction, samples were centrifuged and 1 ml of the supernatant was evaporated and reconstituted with 0.2 ml of EIA buffer provided by the assay kit.

Biochemical validation of the EIA

Cortisol concentrations and the validation tests were determined by using competitive EIA kits (Neogen® Corporation Europe, Ayr, UK). The assay was validated following the criteria for an immunological validation (Reimers & Lamb, 1991). Precision was assessed by calculating intra-assay coefficients of variation (CV) from all duplicated samples analysed. The specificity was evaluated with the linearity of dilution and with the parallelism test. Finally, the sensitivity of the test was given by the smallest amount of hormone concentration analysed.

Statistical analysis

Data obtained were analysed using R software (R-project, Version 3.0.1, R Development Core Team, University of Auckland, New Zealand) with a P-value below 0.05 as a criterion for significance. The assumption of normality was checked using a Shapiro–Wilk test and concentrations were log transformed to achieve normality. An analysis of variance (ANOVA) was used to test for differences between distilled water and isopropanol treatments. When significant, ANOVA was followed by post hoc analysis (Tukey's test) in order to determine the source of significance. For the biochemical validation, statistical correlations in the dilution and parallelism test were determined using the Pearson's Product correlation test.

Results

Washing procedure validation

No significant differences in the scales cortisol concentrations (SCC) were found between samples subject to 1, 2 or 3 washings with isopropanol ($P >$

0.05; Fig. 1a). Whereas samples washed with distilled water showed a significant decrease in the SCC from the first to the second wash ($P < 0.05$), but not from the second to the third one ($P > 0.05$; Fig. 1a). The Tukey's test indicated significant differences in SCC between water and isopropanol when samples were washed twice and thrice ($P < 0.05$).

Significant differences were found in cortisol concentrations between the first, the second and the third wash supernatant of scales treated with isopropanol ($P < 0.05$; Fig. 1b). The same statistically differences were observed in scales washed with distilled water ($P < 0.05$).

Biochemical validation of the EIA

The intra-assay CV was 6.30%. In the linearity of dilution, the obtained cortisol concentrations correlated with the expected cortisol values (r = 0.99, $P < 0.05$; Fig. 2a). In the spike-and-recovery test, hormone standards spiked with the pool of scale extracts presented a mean recovery percentage of 89.59 ± 7.71 % (MEAN ± S.D.). Cortisol concentrations from the standard curve and the pool curve obtained in the parallelism test showed correlation (r = 0.99, $P < 0.05$; Fig. 2b). Finally, the sensitivity of the assay obtained was 0.22 pg of cortisol/mg of scale.

Discussion

Results showed that SCC of samples washed with isopropanol, unlike scales washed with water, remained constant regardless of the number of washings (Fig. 1a). These results could indicate two phenomena: washing scales with isopropanol was not effective enough to remove the external contamination of

cortisol, or water could be penetrating into the scale while removing the hormone deposited inside the matrix. Cortisol concentrations detected in the wash supernatant (Fig. 1b) were actually three orders of magnitude higher than in scales for both isopropanol and water washes, probably due to higher cortisol concentrations found in fish skin mucus (Bertotto et al., 2010). Therefore, the absence of higher cortisol levels detected in scales washed with isopropanol suggests that this solvent was effective in removing the external contamination. Additionally, our results suggest that successive washes with water could be removing endogenous cortisol from the scales, as previously observed in the hair shaft washed with water (Hamel *et al.,* 2011). Accordingly, three 2.5-min isopropanol washings of 3 ml each were established as the washing protocol for the subsequent assessments.

In conclusion, the present work presents a validated methodology to detect cortisol in scales of goldfish. We highlight the importance of an isopropanol washing procedure to completely eliminate external contaminations of cortisol while preserving the inside matrix steroid content. Additionally, we demonstrate the suitability of the EIA in the quantification of cortisol concentrations in scales processed through the aforementioned methodology. The incorporation of this biomarker could provide retrospective hormonal measurements from species and time periods that are usually difficult or impossible to obtain, thus offering key data of an animal's physiology.

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Figure legends

Figure 1. Comparison of distilled water (light grey) and isopropanol (dark grey) mean cortisol concentrations obtained in the validation of the washing procedure: (a) scale cortisol concentrations (SCC) detected in samples subject to 1, 2 and 3 washings and (b) wash cortisol concentrations (WCC) detected in the first, second and third supernatant of each washing procedure. Bars with different letters indicate statistical differences between treatments (Tukey's test, $P < 0.05$).

Figure 2. Results obtained in the biochemical validation of the enzyme immunoassay: (a) correlation between observed and theoretical cortisol concentrations obtained in the dilution test (Pearson's correlation; r = 0.99, $P < 0.05$) and (b) parallelism relation between lines from the standard (white squares) and sample pool (black squares) curves obtained in the parallelism test (Pearson's correlation; r = 0.99, $P < 0.05$).

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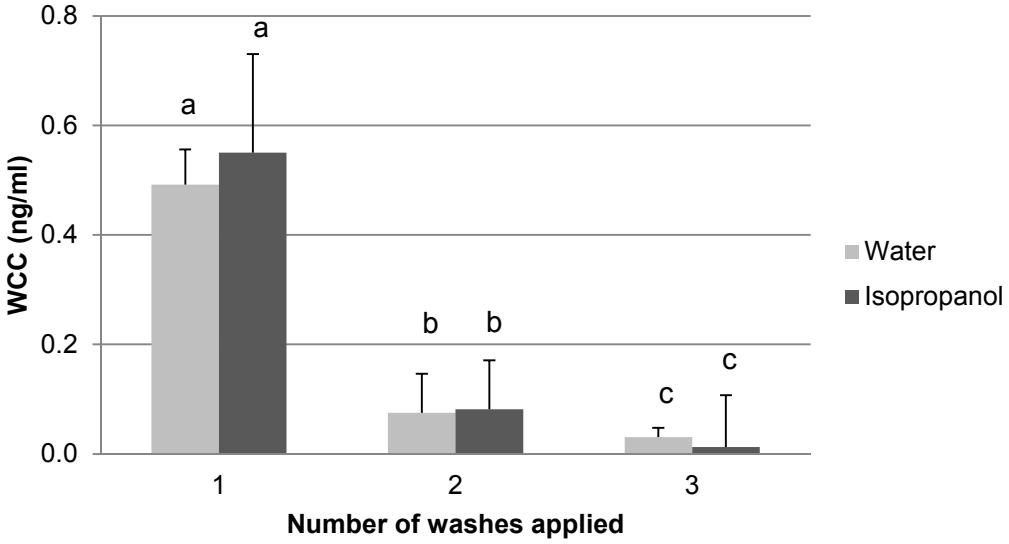

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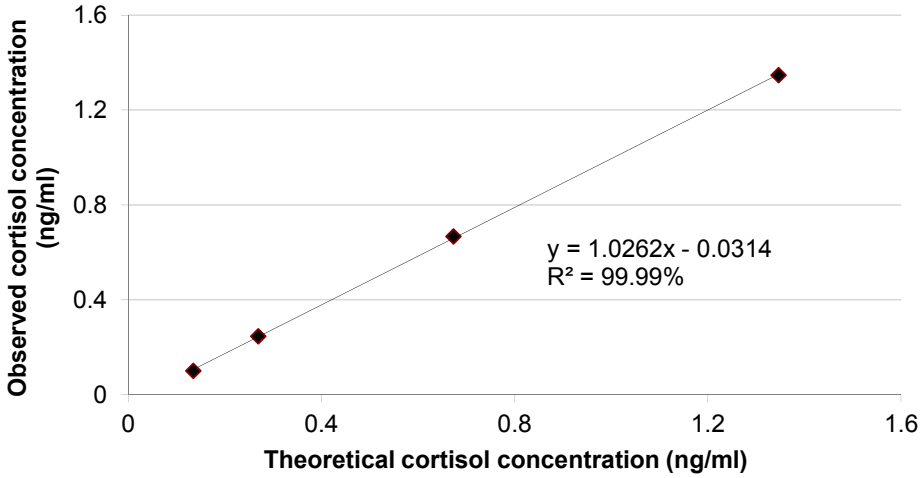

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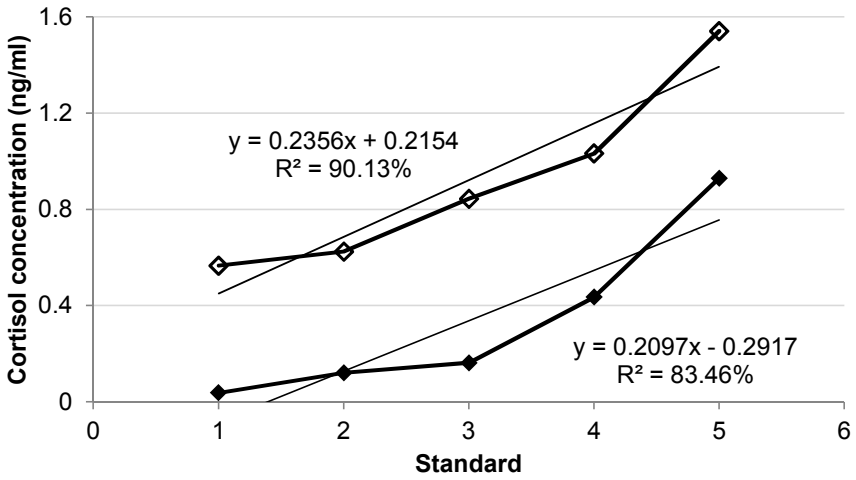

Figure 2. Results obtained in the biochemical validation of the enzyme immunoassay: **(b)** parallelism relation between lines from the standard (white squares) and sample pool (black squares) curves obtained in the parallelism test (Pearson's correlation; $r = 0.99$, $P < 0.05$).