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A simple ultraperformance liquid chromatography mass spectrometry method for the determination of cortisol level in sea bass plasma

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ABSTRACT

A simple, precise, and rapid ultra-performance liquid chromatography method for cortisol quantification in sea bass plasma, based on LC-ESI-UHR-QqTOF-MS, was optimised using tolperisone as internal standard. Sea bass plasma samples containing cortisol were spiked with tolperisone as internal standard and extracted with acetonitrile. Samples were vortexed and centrifuged at 15,000*g* for 10 min at 4 °C. The final extract, diluted in ammonium formate (1:1), was injected in the LC-ESI-UHR-QqTOF-MS system for analysis. Mass spectrometry acquisition parameters were set using positive ionisation mode over a *m/z* range from 150 to 2200. Cortisol and tolperisone were detected and quantified at *m/z* 363.21 and 246.18, respectively, in an Auto MS (MS/MS) mode. The detection and quantification limits of cortisol in sea bass plasma were 0.01 and 0.02 μg/mL, respectively. The mean extraction recovery of cortisol was of 99.1 ± 4.0 %. The within- and between-day precision presents a relative standard deviation (RSD) below 2.9 % and 5.3, respectively. Furthermore, the effect of four different diets (fish feed 1–4) in the basal and stress-induced plasma cortisol levels of sea bass was also assessed.

1. Introduction

Cortisol is an essential hormone that plays important roles, either pathological or physiological, being responsible for the primary response to stress stimulus [14]. In fact, this metabolite is the main glucocorticoid released during the stress response in fish [12], highlighting the importance for its determination. Cortisol is produced in the head kidney and released into the blood, being physiologically active only in its unbound form [12].

The two main methodologies for cortisol determination in biological samples are immunoassays, based on antibodies recognising cortisol [8], and chromatography based analytical techniques, such as liquid chromatography tandem mass spectrometry LC-MS/MS [2]. Furthermore, for the determination of plasma cortisol, preanalytical steps are usually used, typically employing extraction and purification procedures [1,9].

Immunoassays include conventional immunoassays, like radioimmunoassay (RIA), and enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA), but also immunosensors, which are technologically advanced antibody-based assays, or in alternative, molecularly imprinted polymers (MIP)-based biosensors, with signal transduction principles (electrochemical, optical, or others) capable of being combined into portable devices [8]. Routinely, RIA is the most used method for cortisol determination in biological samples [7], nonetheless, is being increasingly replaced by ELISA. These two conventional immunoassays are often the first choice for cortisol determination in plasma, due to their fast and reproducible responses [8]. However, these methods present low selectivity, with possible interferences from other steroids [8] and/or plasma proteins [4]. One particular steroid, cortisone, can be very challenging, since fish can produce considerable amounts of this metabolite [12], and thus interfering with cortisol quantification.

LC-MS/MS is a well-known technique by its specificity and high reliability, despite being an expensive method that requires skilled labour. Nevertheless, it is considered as a reference method for the determination of steroids [11] and is often used to validate immunoassays [12]. In the same way, since LC-MS can separate the various steroids in a single analysis, it manages to overcome possible interferences from this class of metabolites [12].

A well-balanced diet should provide the essential nutrients for the healthy development and growth of fish, but also, if possible, help them

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face stress situations that may occur [10]. Thus, this work aimed at determining the effect of four different diets in sea bass plasma cortisol levels resulting from stress induction. In this context, a simple, precise, and rapid ultra-performance liquid chromatography method for cortisol quantification, based on LC-ESI-UHR-QqTOF-MS, was optimised using tolperisone as internal standard (IS) according to Alvi & Hammami [2].

2. Material and methods

2.1. Stress induction

European sea bass, *Dicentrarchus labrax*, were fed with four commercial-based diets (Table 1) for 12 weeks. Six homogeneous groups of 25 fish (10.8 ± 0.9 g) were randomly distributed among 50 L fiberglass tanks within a saltwater re-circulation system (22 ± 1 °C, 29 %, 3 L/min flow rate, 12 h light/12 h dark light conditions and intensity adjusted to 100 lx at water surface, with twilight transition periods of 30 min). Each diet was randomly assigned to triplicate groups of fish that were fed three times a day (9:00, 12:00 and 16:00) until apparent satiation by automatic feeders. The experimental growth trial, previously approved by the CIIMAR ethical committee for Managing Animal Welfare (ORBEA-CIIMAR_18_2017), was performed by accredited scientists in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGAV-Portugal, following FELASA category C recommendations) and conducted according to the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals for scientific purposes.

At the end of the 12-week period, four fish per tank were collected and sacrificed by anaesthetic overdose (2-phenoxyethanol Sigma-Aldrich, MO, USA) and blood was collected from each fish for further plasma separation and analysis. Additionally, four fish from each tank were subjected to an acute stress challenge. This challenge consisted of a confinement stress protocol designed to simulate overcrowding, followed by air exposure. Four fish per tank were collected and placed in a bucket (100 kg/m^3) with aeration for 5 min, and then exposed to air for 1 min. Following the air exposure, fish were given a 1-hour period for recovery before being netted and euthanised with an overdose of anaesthetic to be sampled. Blood was thus collected at the end of the recovering period. Plasma cortisol was analysed in samples, collected before and after the stress induction, to evaluate the diets potential for fish stress response modulation.

2.2. Seabass plasma preparation

Blood samples (>300 μL blood) were collected from the caudal veins of stressed and non-stressed fish, using 1 mL heparinised syringes and immediately hand mixed to prevent clots. Plasma was then collected after a centrifugation at 5000*g* for 10 min at 4 °C and stored at −80 °C until use.

2.3. Chemicals

Cortisol (hydrocortisone, ≥98 %, H4001) and tolperisone hydrochloride (used as internal standard IS, ≥98 %, T3577) were purchased from Sigma-Aldrich (St. Louis, MO, USA), while acetonitrile (ACN, ≥99 %, LC-MS grade, 83640.911) and ammonium formate (≥99 %, 84884.180) were purchased from VWR (Radnor, Pennsylvania).

Table 1
Diets' characterisation.

	Protein (%)	Lipid (%)	Ashes (%)	Antioxidant Activity(*) (μmol/g)
Fish feed 1	49.3 ± 0.4	16.4 ± 0.1	7.7 ± 0.1	28.1 ± 3.5
Fish feed 2	49.6 ± 0.4	18.5 ± 0.5	7.7 ± 0.1	27.1 ± 5.0
Fish feed 3	49.4 ± 0.1	17.3 ± 0.7	7.7 ± 0.1	27.5 ± 5.0
Fish feed 4	49.3 ± 0.0	19.5 ± 0.4	7.7 ± 0.2	31.0 ± 4.8

(%) dry matter;
(*) μmol Trolox/g of sample.

2.4. Working solutions and standards preparation

Cortisol and tolperisone (Fig. 1) working solutions were prepared in ACN, 25 and 5 μg/mL, respectively. Seven calibration standards were prepared in the range from 0.1 to 2.5 μg/mL in sheep plasma (plasma recovered by centrifugation of sheep blood, defibrinated, SR0051E, Fisher Scientific, Waltham, Massachusetts). Then, aliquots of 500 μL of plasma were transferred to Eppendorf tubes (1 mL) and stored at −20 °C until used.

2.5. Sample extraction

Three proportions of sample extraction with acetonitrile were previously tested (100 + 300, 100 + 400, and 100 + 500) being the best ratio, according to cortisol intensity, 100 μL of the sample plus 400 μL of acetonitrile (results not shown). For this reason, 100 μL of the sample (either from calibration standard or sea bass plasma) were mixed with 10 μL of tolperisone IS-working solution, followed by 400 μL of acetonitrile for protein precipitation. Samples were vortexed (10 s) and centrifuged at 15,000*g* for 10 min at 4 °C. Then, an aliquot of 100 μL of supernatant was diluted with 100 μL of 10 mM ammonium formate. The final extract was transferred to a glass vial with insert, and 10 μL of the mixture were injected to the LC-ESI-UHR-QqTOF-MS system for analysis.

2.6. Instrumental and chromatographic conditions

An UHPLC from Bruker Elute series, coupled to an UHR-QqTOF mass spectrometer (Impact II, Bruker) was used. Separation of plasma metabolites was performed using a BRHSC18022100 intensity Solo 2 C18 column (100 × 2.1 mm, 2.2 μm, Bruker) using water plus 0.1 % formic acid (mobile phase A) and acetonitrile plus 0.1 % formic acid (mobile phase B) with a gradient flow of 0.250 mL/min. The mobile phase gradient was optimized as follows: 0–2 min (99 % A), 2–5 min (99–25 % A), 5–9 min (25–0 % A), 9–11 min (0 % A), 11–12 min (0–25 % A), 12–13 min (25–99 % A), and finally (99 % A) during the last 2 min (13–15 min). The mobile phase effect on both resolution and retention time of cortisol was previously studied. Two additional gradients were tested. The first, from 0 to 1 min (99 % A) and from 1 to 5 min (99–25 % A), and the second, from 0 to 5 min (99–25 % A). From minute 5 it was applied the same gradient as the used methodology (results not shown).

All samples were acquired in positive ionisation mode using an ESI source (Table 2) in Auto MSMS scan mode, where the most predominant MS ions were chosen for MS2 fragmentation. Auto MSMS settings are present in Table 3. Post-acquisition internal mass calibration used a sodium formate solution delivered by a syringe pump at the start of each chromatographic analysis.

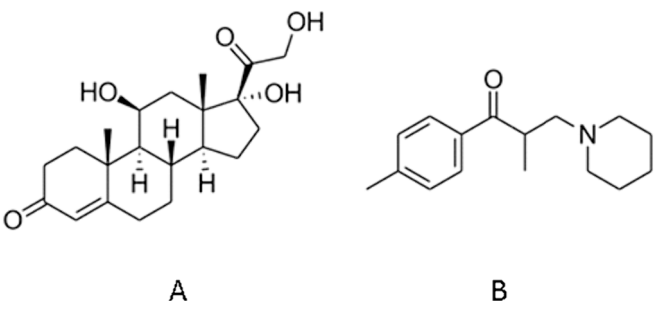

Fig. 1. Chemical structure of cortisol (A) and tolperisone (B) (IS).

Table 2
ESI source parameters.

	Positive mode
End plate offset voltage (V)	500
Capillary voltage (V)	4500
Drying gas temperature (°C)	200
Drying gas flow (L/min)	8.0
Nebulizing gas pressure (bar)	2.0

Table 3
Auto MSMS settings.

	Full scan MS
Mass range (*m/z*)	20–1000
Absolute threshold (cts)	14
Spectra rate (Hz)	1.0
	MSMS
Mass range (*m/z*)	20–1000
Absolute threshold (cts)	303
Max precursor per cycle	5
Spectra rate (Hz)	1.0
Collision energy (eV)	20–50

2.7. Accuracy

Accuracy was determined as % of extraction recovery.

2.8. Extraction recovery

Extraction recovery of cortisol was measured by comparing cortisol concentration in two different samples spiked with the same amount of cortisol: stressed (higher-level) and non-stressed (lower-level) samples. The recovery was also carried out using a blank plasma sample with the addition of three different amounts of cortisol. The study was carried out by spiking the samples with cortisol prior to extraction. The procedure was performed in three replicates, and the extraction recovery calculated according to Eq. (1) [3].

$$R(\%) = \frac{[Cortisol](recov)}{[Cortisol]\,(orig) + [Cortisol](added)} \times 100 \quad (1)$$

where, $[Cortisol](recov)$ is the recovered quantity of the cortisol, $[Cortisol](orig)$ is the original concentration of cortisol, and $[Cortisol](added)$ is the amount of cortisol added for the recovery.

2.9. Precision

The within-day and between-day precision was accessed by the percentage in relative standard deviation (RSD %) for n = 3. The procedure was carried out for samples with different cortisol levels: stressed (higher-level) and non-stressed (lower-level) samples.

2.10. Method validation

The method was validated (specificity, linearity, accuracy, recovery, and precision) according to European Medicines Agency [6].

2.11. Statistical analysis

Results are presented as mean ± standard deviation. Statistical evaluation of tests was carried out using two-way ANOVA, using Statistica Software Version 14.0.1.25.

3. Results and discussion

3.1. Identification of cortisol and internal standard

Cortisol and tolperisone (internal standard) were detected in Auto MSMS scan mode. Both full scan MS and MS2 spectrums were acquired. Identification of internal standard and cortisol was performed at *m/z* 246 and *m/z* 363, respectively. Fig. 2A represents a sea bass plasma sample, while Fig. 2B represents a blank plasma spiked with internal standard. In both Figures, extracted ion chromatograms (EIC) are show for *m/z* 246 (1) and *m/z* 363 (2). In the sea bass plasma sample, full MS and MS2 spectrums were acquired for both internal standard, at 5.4 min, and cortisol, at 5.7 min (Fig. 2A). In the blank plasma spiked with internal standard sample, full MS and MS2 spectrums were acquired for internal standard, at 5.4 min, but no peak was found for cortisol, at 5.7 min, in full MS (Fig. 2B). Cortisol fragmentation path was confirmed by the analytical commercial standard according to [15].

3.2. Specificity

The specificity of the assay was determined by the screening of seven different batches of blank plasma with two different hormones/neuromodulators (dopamine, serotonin) and a serotonin metabolite (5-hydroxyindoleacetic acid). No interference with the peaks of cortisol or IS was observed.

3.3. Linearity and limit of detection and quantification

The linearity of the method was defined by the analysis of cortisol standards at seven different concentrations over the range of 0.01–2.5 μg/mL. Peak areas ratios of cortisol with internal standard and cortisol concentrations were exposed to regression analysis. Mean calibration curves obtained from three individual standard curves within- and between-days were achieved. The obtained mean equations (Y = ax + b) where: Y = 0.313 ± 0.005 (x) + 0.004 ± 0.001 (r^2 = 0.999 ± 0.000) for within-days; and Y = 0.299 ± 0.015 (x) + 0.005 ± 0.003 (r^2 = 0.998 ± 0.001) for between-days. The slopes, intercepts, and coefficients of determination, obtained for within- and between-days calibration curves, were not significantly different ($p > 0.05$; 95 % confidence interval). The mean equation obtained for the six standards curves is presented in Table 4. The limit of detection (LOD) and limit of quantification (LOQ) were determined follow the Eqs. (2) and (3), respectively.

$$LOD = \frac{3Sy/x}{a} \quad (2)$$

$$LOD = \frac{10Sy/x}{a} \quad (3)$$

where $S_{y/x}$ is the instrumental signal value dispersion around the calibration curve and a is the slope of the calibration curve.

3.4. Extraction recovery

The extraction recovery results obtained are presented in Table 5.

Considering all the analysed samples, the mean extraction recovery was of 99.1 ± 4.0 %. By using a blank plasma sample, it was possible to carry out the analysis at three different levels of addition (Table 4).

3.5. Precision

The within-day and between-day precision results are presented in Table 6. As it can be observed, the within-day precision presents a relative standard deviation (RSD) below 2.9 and 1.9 % for the lower and higher-level samples, respectively. Considering the between-day precision, it was possible to obtain RSD below 5.3 and 3.7 %, for the lower and higher-level samples, respectively.

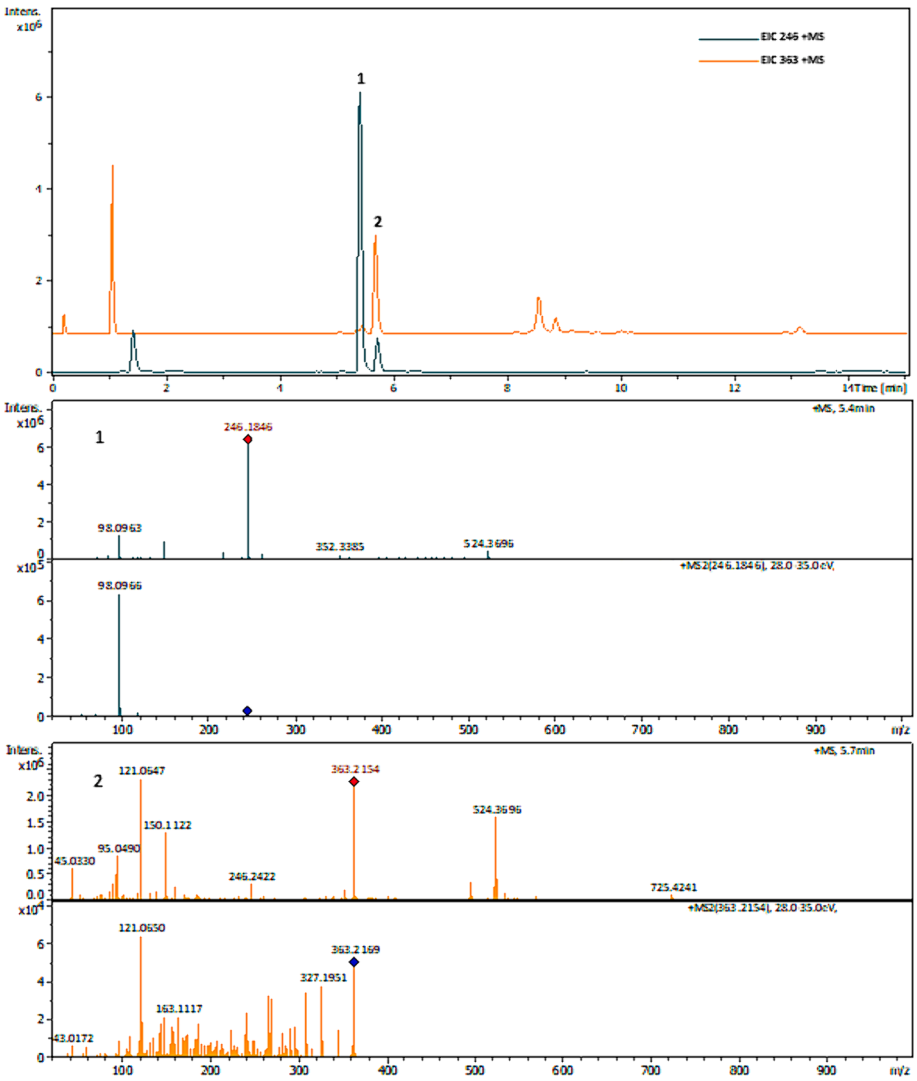

Fig. 2A. Extracted ion chromatograms and respective MS and MS2 spectrums in sea bass plasma for: [1] tolperisone (EIC 246 + MS) and [2] cortisol (EIC 363 + MS).

3.6. Plasma analysis

The developed method was used to determine plasma cortisol in sea bass samples from stressed and non-stressed fish fed with different diets (Table 7). The stressed fish consistently had higher cortisol levels than non-stressed fish ($p = 0.0000$), regardless of the dietary treatment, with overall averages of 0.65 ± 0.09 and 0.30 ± 0.08 μg/mL in stressed and non-stressed samples, respectively. This variation of cortisol concentration between the basal and post-acute stress conditions is consistent with several studies [13]. Moreover, the variation of cortisol levels, in European sea bass can occur due to irregular body size, genetics, coding styles, temperature, salinity, photoperiod, season, or even food composition [5]. Indeed, the obtained results showed significant differences in cortisol levels in fish subject to different type of feed ($p = 0.0001$). Fish fed with diet 1 and 2 presented significantly higher cortisol levels than fish fed with diet 3 and 4, for both stressed and non-stressed fish (Table 7).

4. Conclusions

A simple, precise, and rapid ultra-performance liquid chromatography method for cortisol quantification in sea bass plasma, based on LC-ESI-UHR-QqTOF-MS, was optimised using tolperisone as internal standard. The slopes, intercepts, and coefficients of determination obtained for within- and between-days calibration curves were not significantly different ($p > 0.05$; 95 % confidence interval), and the detection and quantification limits of cortisol in sea bass plasma were 0.01 and 0.02 μg/mL, respectively. The mean extraction recovery of cortisol in the optimised method was of 99.1 ± 4.0 %, and the within- and between-day precision presents a relative standard deviation (RSD) below 2.9 and 5.3 %, respectively. Furthermore, the method was successfully applied to determine cortisol in sea bass plasma of stressed and non-stressed fish, allowing to determine that fish feed could modulate the stress response.

Statement of novelty

A well-balanced diet should provide the essential nutrients for the healthy development and growth of fish, but also, if possible, help them face stress situations that may occur. In this way, the determination of the effect of different diets in the production of cortisol resulting from stress induction is very important. Many methodologies used for plasma cortisol quantification are based in immunoassays with many interferences. In this context, a simple, precise, and rapid ultra-performance liquid chromatography method for cortisol quantification in sea bass plasma, based on LC-ESI-UHR-QqTOF-MS, was optimised.

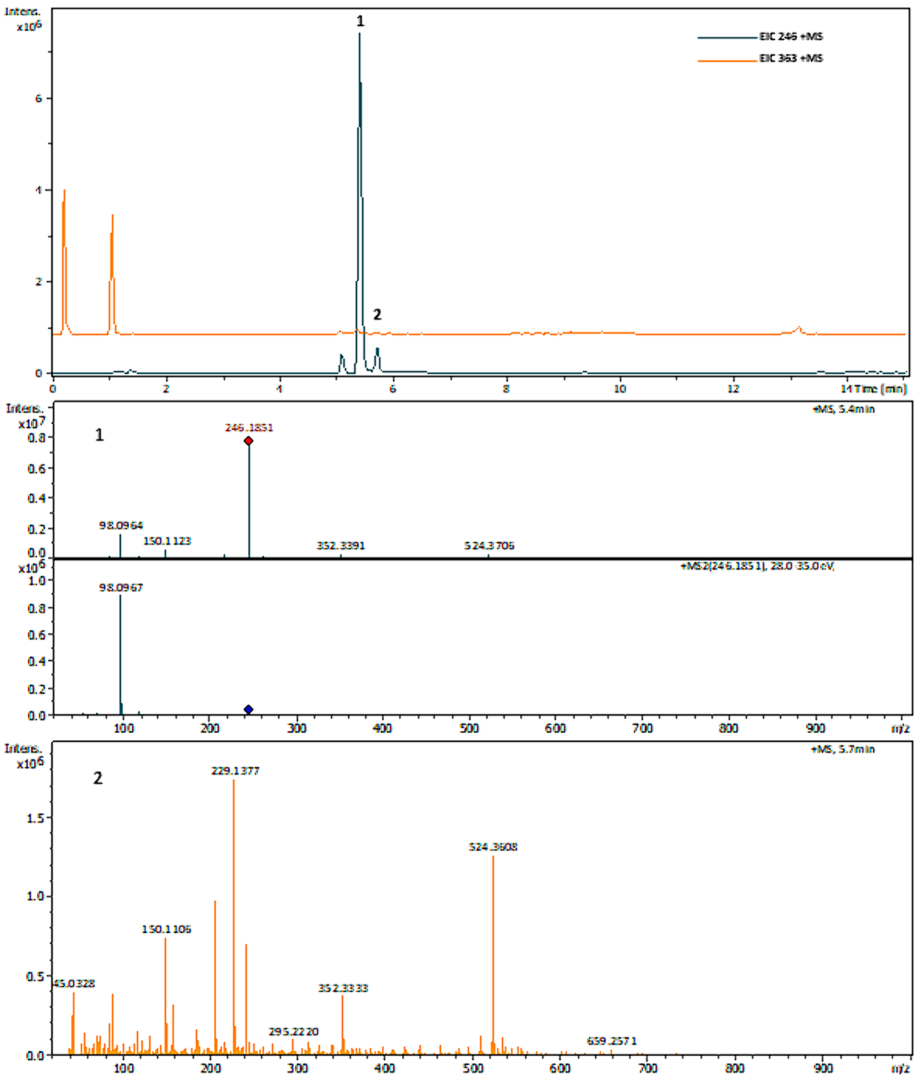

Fig. 2B. Extracted ion chromatograms and respective MS and/or MS2 spectrums in blank plasma spiked with internal standard for: [1] tolperisone (EIC 246 + MS) and [2] cortisol (EIC 363 + MS).

Table 4
Linear regression parameters.

	Linear range (μg/mL)	Slope (a)	Intercept (b)	r^2	LOD (μg/mL)	LOQ (μg/mL)
Cortisol	0.01–2.5	0.306 ± 0.010	0.004 ± 0.002	0.998 ± 0.000	0.01	0.02

r^2 = coefficient of determination; LOD = Limit of detection; LOQ = Limit of quantification.

Table 5
Extraction recovery of cortisol from plasma samples.

Cortisol added (μg/mL)	Recovery (%)		
	Blank plasma	Lower-level	Higher-level
0.125	100.6 ± 2.6	103.7 ± 0.8	101.2 ± 1.0
0.25	91.1 ± 2.4	100.4 ± 0.5	–
0.50	97.4 ± 2.2	–	–

Higher-level: stressed sample; Lower-level: non-stressed sample.

Table 6
Within-day and between-day precision.

Sample	Within-day precision (RSD, %)	Between-day precision (RSD, %)
Lower-level 1	0.7	3.3
Lower-level 2	2.9	2.9
Lower-level 3	1.5	5.3
Higher-level 1	1.3	3.7
Higher-level 2	0.2	3.4
Higher-level 3	1.9	2.0

RSD, Relative standard deviation; n = 3.

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CRediT authorship contribution statement

Carla M. Oliveira: Writing – review & editing, Validation,

Table 7
Cortisol concentration in the stressed and non-stressed samples.

	Condition	Cortisol (μg/mL)
Fish Feed 1	Stressed	0.72 ± 0.11^{a}
	Non-stressed	0.35 ± 0.10^{a}
Fish Feed 2	Stressed	0.78 ± 0.10^{a}
	Non-stressed	0.32 ± 0.07^{a}
Fish Feed 3	Stressed	0.58 ± 0.07^{b}
	Non-stressed	0.28 ± 0.08^{b}
Fish Feed 4	Stressed	0.52 ± 0.10^{b}
	Non-stressed	0.24 ± 0.06^{b}
Two-way ANOVA p-value	Diet	0.0001
	Stress	0.0000
	Diet * Stress	0.0821

Values are presented as mean ± standard deviation (n = 3). Values in the same row without a common superscript letter differ significantly ($p < 0.05$).

Supervision, Methodology. **Inês Campos:** Writing – review & editing, Investigation, Conceptualization. **Susana S.M.P. Vidigal:** Writing – original draft, Investigation, Conceptualization. **Manuela E. Pintado:** Funding acquisition. **Catarina S.S. Oliveira:** Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

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