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Development of a microtitre plate indirect ELISA for measuring cortisol in teleosts, and evaluation of stress responses in rainbow trout and gilthead sea bream

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A microtitre plate indirect enzyme-linked immunoassay (ELISA) was developed for measuring plasma cortisol levels in rainbow trout *Oncorhynchus mykiss*, gilthead sea bream *Sparus auratus* sea bass *Dicentrarchus labrax* and Senegalese sole *Solea senegalensis*. Covalink microplates pretreated with disuccinimidyl suberate were coated with bovine serum albumin (BSA) conjugated to cortisol-3-carboxymethyl oxime. After blocking with BSA, competition was started by addition of plasma samples and anti-cortisol antibody raised in rabbit. Goat anti-rabbit IgG conjugated-peroxidase was added as second antibody and then incubated with orthophenylenediamine as substrate. Reaction was stopped with 0·1 M HCl and absorbance was read at 450 nm in an automatic plate reader. The standard curve was linear from the lower limit of sensitivity of the assay (*c.* 0·3 ng ml^{-1}) to *c.* 3000 ng ml^{-1}. Dose-response inhibition curves using serially diluted plasma samples of four species consistently showed parallelism with the standard curve using cortisol. The ELISA satisfied the strictest criteria of specificity (cross-reactivity of anti-cortisol antibody with testosterone, progesterone and 17ß-oestradiol was negligible, cross-reactivity with cortisone, corticosterone and 11-deoxycortisol, was 1·5, 1 and 0·1%, respectively), reproducibility (interassay CV <6%), precision (intra-assay CV <4%), and accuracy (average recovery >98%). Plasma cortisol concentration in rested fishes was in the range of 5–30 ng ml^{-1}. To physiologically validate the technique, changes in plasma cortisol concentrations were also measured in plasma of rainbow trout and gilthead sea bream following an acute 15 min chasing or 3 min air-exposure stress, respectively. In both species plasma concentrations of cortisol, glucose and lactate rose significantly with respect to controls, showing concentrations similar to those reported previously for these species under similar stress conditions. Furthermore, gilthead sea bream chronically stressed by maintaining for 14 days under increased stocking density conditions also showed increased concentrations of plasma cortisol and glucose. These results validate the indirect ELISA technique developed for use in the evaluation of plasma cortisol concentration of at least four fish species.
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Key words: cortisol; ELISA; gilthead sea bream; rainbow trout; sea bass; Senegalese sole.

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INTRODUCTION

Stress responses in teleosts involve primary, secondary and tertiary responses. An effective way to assess primary response is to measure changes in plasma cortisol concentration (Barton & Iwama, 1991; Wendelaar Bonga, 1997). A drawback of this approach is that its use has generally been limited to research laboratories which have the capability of measuring cortisol by radio-immunoassay (RIA). An alternative assay method, that avoids the use of radioactivity, for monitoring cortisol concentration in fishes is enzyme-linked immunosorbent assay (ELISA). Accordingly, several direct (*i.e.* antibody is coated to the plate) ELISA methods have been validated for use in fish species (Barry *et al.*, 1993; Nielsen *et al.*, 1994; Carey & McCormick, 1998; Kelly & Woo, 1999). Another more simple indirect ELISA method involved coating the plate with antigen instead of antibody (Crowther, 2001). As far as is known, no indirect ELISA has been set up for the measurement of plasma cortisol concentration in fishes.

The purpose of the present study was to develop and validate a microtitre plate for indirect ELISA to measure plasma cortisol concentration in several species of well known cultured teleosts such as rainbow trout *Oncorhynchus mykiss* (Walbaum), gilthead sea bream *Sparus auratus* (L.), sea bass *Dicentrarchus labrax* (L.) and Senegalese sole *Solea senegalensis* Kaup. Moreover, to physiologically validate the method, two of those species (rainbow trout and gilthead sea bream) were submitted to several types of stress known to increase plasma cortisol concentration.

MATERIALS AND METHODS

ANIMALS AND EXPERIMENTAL DESIGN

Immature male gilthead sea bream (100–150 g body mass), Senegalese sole (200–250 g) and sea bass (15–20 g) were provided by Planta de Cultivos Marinos (CASEM, Universidad de Cádiz, Puerto Real, Cádiz, Spain) and transported to the laboratories at Faculty of Marine Sciences Puerto Real, Cádiz, Spain where experiments were carried out. Fishes were acclimated to sea water in 1000 l tanks for at least 2 weeks in an open system (salinity 38, 1000 mOsm kg^{-1} H_2O). During the experiments, fishes were maintained under natural photoperiod (April; 42° 14′ N) and temperature (19–21° C). Fishes were fed daily at 1% body mass using commercial dry pellets (Trouvit Europa D-5, Trout España, Burgos, Spain), which constituted a maintenance diet for this species. They were fasted for 24 h before experiments.

On day of sampling, six Senegalese sole and six sea bass were anaesthetized with 2-phenoxy ethanol (0·1%). In Senegalese sole, blood was taken from the caudal peduncle using ammonium-heparinized syringes, while in sea bass blood was obtained by severing the tail and collecting the blood in ammonium-heparinized microcapilary tubes. Plasma was obtained after centrifugation, aliquoted and frozen in liquid nitrogen for subsequent assessment of concentrations of cortisol, glucose and lactate.

Gilthead sea bream were used to assess basal concentration of cortisol as well as to assess the effects of short- and long-term stress conditions on plasma cortisol concentration. Thus, in a first experiment, fish were submitted to a short-term stress by air exposure according to the method described by Arends *et al.* (1999) that significantly elevated plasma cortisol concentration. Fish were randomly distributed into two groups and placed in two tanks containing a plastified iron wire-net cage. One group (eight fish) was acutely stressed by exposing them to air during 3 min by lifting the wire-net out of the water, after which the cages were put back into the tank. The other group (eight fish)

remained in the tank and served as controls. After 20 min both groups were anaesthetized to obtain blood samples using ammonium-heparinized syringes as described above. In a second experiment, fish were submitted to long-term stress by increasing the stocking density according to the method used by Arends *et al.* (1999). Fish were randomly distributed in four different 300 l tanks containing a plastified iron wire-net cage with a total volume of 250 l (inner diameter of cage 0·60 cm) to obtain a fish density of 4 kg m^{-3}. The fish were allowed to acclimate to the experimental tanks for 7 days. After acclimatization, the wire-net cage in the tank were raised (water depth *c.* 15 cm) to increase the stocking density from 4 to 15, 30 or 70 kg m^{-3}, whereas one group remained in one tank at 4 kg m^{-3} and served as control. After 14 days, eight fish from each stocking density were removed by dip-net and sampled as described above. No mortality was observed during the experiments.

Rainbow trout (100–110 g body mass) were obtained from a fish hatchery (Soutorredondo, Spain) and maintained in the Faculty of Marine Sciences in Vigo (Spain) for 1 month in 100 l tanks under laboratory conditions and natural photoperiod (May) in dechlorinated tap water at 13° C. The fish were fed daily to satiation with a standard Purina trout chow and were deprived of food the day before used in experiments. On the day of the experiment, one group (10 fish) in a tank were acutely stressed by chasing in the tank during 15 min, whereas another group (10 fish) maintained in another tank served as controls. Fish were dip-netted and anaesthetized with 2-phenoxy ethanol (0·1%). Plasma samples were obtained using ammonium-heparinized syringes as described above.

All experiments described comply with the Guidelines of the European Union Council (86/609/EU), and of the Universities of Cádiz and Vigo (Spain) for the use of laboratory animals.

ASSAY OF PLASMA GLUCOSE AND LACTATE

Plasma glucose and lactate were measured using commercial kits from Spinreact (Spain) adapted to microplates.

INDIRECT ELISA FOR CORTISOL ASSAY

Reagents and solutions

Composition of solutions used was as follows: 1) bovine serum albumin (BSA) solution: 83 mg of BSA dissolved in a mixture of 2·5 ml of water and 42 µl of 1 M NaOH, followed by the slow addition of 0·83 ml of dimethylformamide; 2) assay buffer: 0·15 mol l^{-1} phosphate buffer, 0·15 mol l^{-1} NaCl (pH 7·2); 3) coating buffer: 0·532 mmol l^{-1} disuccinimidyl suberate (DSS) in assay buffer (DSS was previously dissolved in dimethyl sulphoxide, DMSO); 4) blocking solution: 0·1% BSA in assay buffer; 5) washing buffer: 0·05% tween 20 in assay buffer; 6) anti-cortisol antibody solution: one vial of liophilized anti-cortisol whole antiserum developed in rabbit (Sigma, C-8409) was dissolved in 0·05 mol l^{-1} tris-HCl buffer (pH 8·0) containing 0·1 mol l^{-1} NaCl, 0·1% gelatine, and 0·1% sodium azide; this stock solution was diluted (see below) with assay buffer to make up a working anti-cortisol antibody solution; 7) substrate solution: one tablet of orthophenylenediamine dihydrochloride (OPD; Sigma, P-9187) dissolved in 20 ml of water; 8) stop solution: 0·5 mol l^{-1} HCl; 9) charcoal stripped-plasma: prepared by adding 50 mg of charcoal to 1 ml of plasma, stirring for 1 h at 20° C, leaving overnight at 4° C, centrifuging the supernatant and filtering through 0·45 µm filter (Whatman); 10) cortisol standards: a stock solution of cortisol was made in ethanol and standards were diluted in Ringer's solution after comparison with charcoal stripped plasma standards.

Preparation of cortisol-BSA conjugate

Carboxylic acid derivatives of cortisol were prepared following the methods of Hosoda *et al.* (1979) and Asahina *et al.* (1995) with some modifications.

Preparation of cortisol-3-(O-carboxymethyl)oxime (Cortisol-3-CMO): 25 mg of cortisol was dissolved in a mixture of 100 μl chloroform and 300 μl methanol. Sodium acetate and carboxymethylmethoxylamine, 12·5 mg each, were dissolved in a mixture of 20 μl water and 20 μl methanol, and were added to the cortisol solution at room temperature. After completion of the conversion, *c.* 50 min after the start of the reaction, 3 ml of water were then added to stop the reaction. Extraction was performed twice by adding 10 ml of ethyl acetate and shaking vigorously. The combined ethyl acetate phases were washed twice with water, dried over anhydrous Na_2SO_4, and evaporated under a gentle stream of nitrogen.

Preparation of cortisol-3-(O-carboxymethyl)oxime N-hydroxysuccinimide ester (Cortisol-3-CMO ester): 25 mg of cortisol-3-CMO, 20 mg of N-hydroxysuccinimide, and 30 mg of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide-HCl were mixed and dissolved in 250 μl of dimethylformamide and left at room temperature for 1 h. After completion of the conversion, the reactant was extracted twice with 10 ml of ethyl acetate, and the pooled solvent was washed twice with 10 ml of water. The ethyl acetate fraction was dehydrated with anhydrous Na_2SO_4, and evaporated under a gentle stream of nitrogen.

Formation of the conjugate of cortisol-3-(O-carboxymethyl)oxime N-hydroxysuccinimide ester with BSA (Cortisol-3-CMO-BSA): the active ester of cortisol-3-CMO was conjugated to BSA using a 30 : 1 molar ratio of steroid to BSA. BSA solution was mixed with a solution of cortisol-3-CMO ester (25 mg dissolved in 0·5 ml of dimethylformamide). After 30 min of stirring, the reactant was dialyzed against cold running water overnight, and the solution was brought to pH 4·5 with 1 N HCl, and centrifuged at 4000 *g* for 15 min, and the dyalisis was repeated until the free steroid and N-hydroxysuccinimide were removed completely. The precipitate was dissolved in water adjusted to pH 7·0 with 1 N NaOH, diluted to a concentration of 10 mg protein ml^{-1}, and stored at −80° C. The concentration of protein was assessed as described by Bradford (1976).

Determination of the effectiveness of conjugation: ultraviolet spectral measurements were carried out on a Unicam UV6–220 spectrophotometer (Thermo Unicam, Waltham, MA, U.S.A.). The solvent used was 0·05 mol l^{-1} phosphate buffer (pH 7·4) or 0·1 mol l^{-1} KOH. Spectral analysis and calculations were carried out as described by Hosoda *et al* (1979) by comparing the absorbance (molar extinction coefficient of 23560) of the conjugate cortisol-3-CMO ester with BSA with those of BSA, cortisol and cortisol-3-CMO ester alone in both solvents.

Assay procedure

A 50 μl solution of coating buffer was added to the wells of Covalink 96-well microtiter plates (Nunc, Roskilde, Denmark). The plate was covered with an adhesive plate-sealer and incubated overnight at 4° C. The unbound DSS was removed by shaking out and washing each well three times with 0·2 ml of water. The plate was then dried by inverting it on a paper towel for several minutes and stored at 4° C until use.

The DSS-pretreated plates were coated with 50 μl of a solution containing the conjugate of cortisol-3-CMO with BSA (the concentration of the solution was adjusted to give *c.* 5000 ng cortisol ml^{-1}) or BSA (0·09 mg ml^{-1}, the same concentration of BSA as used in the cortisol-3-CMO conjugate with BSA, to determine non-specific binding). Plates were left overnight at 4° C.

Plates were incubated for 1·5 h at 37° C with 150 μl of blocking solution per well. Then, each plate was shaken out and wells were washed four times with 200 μl of washing buffer. The plate was then dried by inverting it on a paper towel for several minutes.

Competition was started by adding to the plates 50 μl of standard solutions or unknown samples, then 50 μl of anti-cortisol antibody solution at a final dilution in wells of 1/5 (v/v), and left 2·5 h at 18° C. The unbound first antibody was removed by shaking out and washing each well five times with 200 μl of washing buffer. The plate was then dried by inverting it on a paper towel for several minutes and using it for the next step of the ELISA.

A 50 μl solution of goat anti-rabbit IgG conjugated-peroxidase (Sigma; final dilution of 1/500 in assay buffer) was added to all wells and incubated 2·5 h at 18° C. The unbound second antibody was removed by shaking out and washing each well five times with 200 μl of washing buffer. The plate was then dried by inverting it on a paper towel for several minutes and used for the colour reaction.

Each well received 100 μl of freshly prepared substrate solution and was left for 30 min at 20° C. The reaction was stopped by adding 100 μl of stop solution and absorbance was read at 450 nm in an automatic plate reader (SpectraFluor, Tecan, Zurich, Switzerland).

Expression of results

A standard curve was performed for each plate using concentrations ranging from 0·03 to 30 000 ng cortisol ml^{-1}. On each plate, three wells received only OPD substrate to estimate the reagent blank, three wells were used to measure the non-specific binding (NSB), and the other three wells were used to assess the maximum binding (Bo).

The specific binding (Bi) for each standard or sample was expressed by the difference between total optical density (measured in each conjugate-coated well) and non-specific optical density (from BSA-coated wells), and Bo represented the maximum specific binding to the plate. The Bi:Bo ratios were used to draw the standard curve, which was converted into a logit-log representation to yield a linear relationship by the linear square method. This representation was used to estimate the cortisol concentration in each sample.

Reproducibility, precision, accuracy and specificity of the assays

To assess the reproducibility (measured by interassay CV) and precision (measured as intra-assay CV) of the assay, several pooled plasmas and different standard solutions were measured in different conditions. Samples and standards were assayed eight times in the same set of experiments, and in six different series.

To test the accuracy of the assays (overloading tests), replicates ($n = 5$) of known amounts of cortisol were added to aliquots of charcoal-stripped plasma. Aliquots were assayed following the normal procedure, and the linear regressions from measured cortisol *v.* added cortisol were calculated.

The specificity of the ELISA was tested by cross reactivity of the anti-cortisol antibody with different steroids. The ELISA was carried out as described above using serial dilutions of different steroids besides that of cortisol in the standard wells. The cross-reactivity of the antibody was tested with 11-deoxycortisol, cortisone, corticosterone, progesterone, 17β-oestradiol and testosterone, and calculated as the quantity of the competitor antigen that resulted in 50% inhibition in the binding of the tracer and expressed as a percentage.

STATISTICAL ANALYSIS

Data on hormone or metabolite concentrations in the different experiments with fishes were expressed as means ± S.E. and analysed by one way ANOVA followed by Student Newman Keuls multiple range test (density experiment with gilthead sea bream) of by a *t*-test (acute stress in gilthead sea bream and rainbow trout). The differences were considered statistically significant at $P < 0{\cdot}05$.

RESULTS

ELISA VALIDATION

The optimal assay conditions for cortisol-3-CMO-BSA conjugate and the respective rabbit anti-cortisol were obtained by incubation of serial dilutions of both tracer and antibody resulting in the coating concentration, primary

antibody dilution and secondary antibody dilution. Under these conditions, the maximum optical densities (Bo) were found to be *c.* 1·7, which were in the range of the linear response of the plate reader. The optical densities of non-specific binding wells at these tracer concentrations were *c.* 1·1.

A typical dose-response curve of cortisol standards dissolved in Ringer's solution is shown in Fig. 1(a). Curves generated using charcoal-stripped plasma from rainbow trout, gilthead sea bream, Senegalese sole or sea bass were indistinguishable from that of standards dissolved in Ringers'solution. Serially-diluted pooled plasma samples from four species were parallel to the standard curve, and all dilutions fell within the linear portion of the standard curve [Fig. 1(b)].

The sensitivity of the assay was *c.* 0·3 ng ml^{-1}. The precision of the assay, determined by calculating the intra-assay CV of repeated measures of samples within the same assay was <4%, regardless of whether the samples contained different concentrations of cortisol. There were no species differences.

The reproducibility of the assay, calculated as the interassay CV, was determined by measuring the same samples in six separate assays. The average inter-assay CVs of samples were always <6%, and no species differences were detected.

The accuracy of the ELISA was calculated as the per cent of exogenous cortisol recovered from spiked plasma. The overall recovery was >98%. A linear regression line fitted through these data gave an r^2 value of 0·99. Similar results

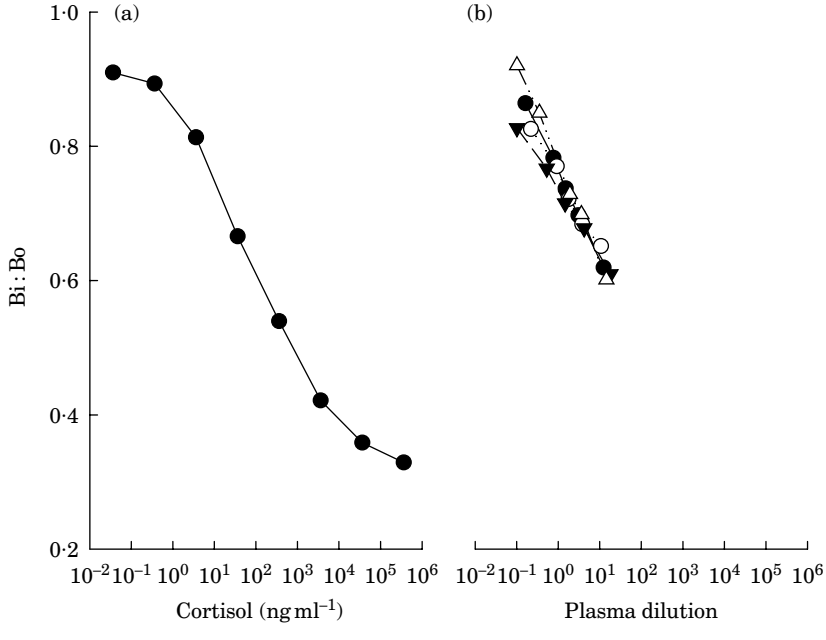

FIG. 1. (a) Displacement curves of cortisol standards and (b) serial dilutions of plasma from gilthead sea bream (●), rainbow trout (○), sea bass (▼), and Senegalese sole (△). Each point is the mean of eight determinations for cortisol standards, and three for plasma dilutions. Bi, specifically bound tracer; Bo, maximum bound tracer.

were obtained using plasma from the four different species used (gilthead sea bream, Senegalese sole, sea bass and rainbow trout).

The specificity was assessed by cross-reactivity of different steroids to the anti-cortisol antibody as displayed in Fig. 2. Testosterone, progesterone and 17ß-oestradiol showed negligible cross-reactivity. Cortisone, corticosterone and 11-deoxycortisol had 1·5, 1, and 0·1% of cross-reactivity, respectively.

STRESS RESPONSES

Basal plasma concentrations of cortisol, glucose and lactate in the different fish species are given in Table 1.

In rainbow trout, the short-term treatment of chasing fish in the tank for 15 min induced a significant increase in plasma concentrations of cortisol, glucose and lactate (Table 1). In gilthead sea bream another short-term treatment, air exposure for 3 min, induced a very sharp increase in plasma cortisol concentration and also an increase in plasma glucose and lactate concentrations (Table 1).

The long-term acclimation of gilthead sea bream to increased stocking densities induced several changes in variables measured in plasma (Table II). Thus, cortisol concentration significantly increased in fish acclimated to stocking densities of 30 and 70 kg m^{-3} compared with fish acclimated to densities of 4 and 15 kg m^{-3}. Plasma glucose concentration displayed a similar trend with values

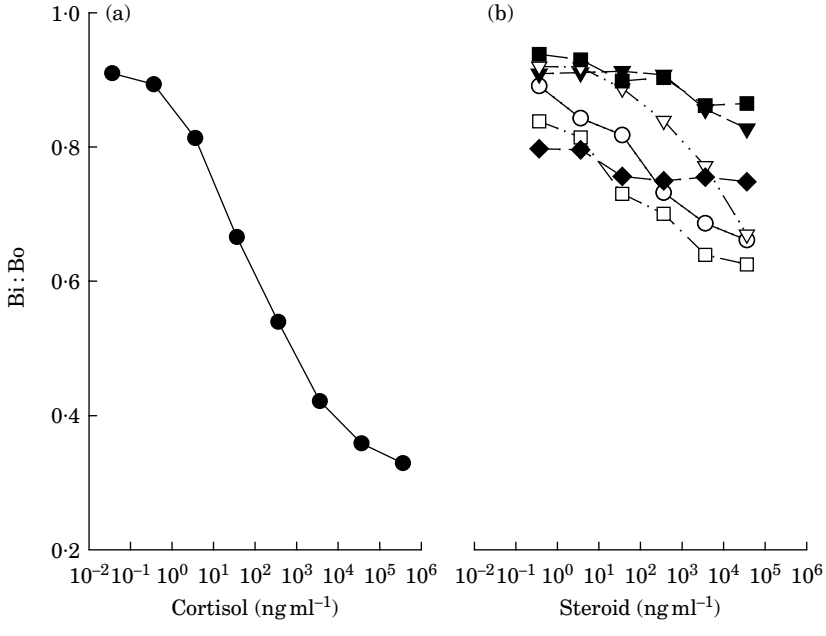

FIG. 2. (a) Displacement curves of cortisol standards, and (b) dose-response inhibition curves of different steroids (●, cortisol; ○, 11-deoxy cortisol; ▼, progesterone; ▽, corticosterone; ■, 17ß-oestradiol; □, cortisone; ◆, testosterone) used to determine specificity of antibody. Each point is the mean of eight determinations for cortisol standards, and three for other steroids.

TABLE I. Plasma levels of cortisol, glucose and lactate in rainbow trout, gilthead sea bream, Senegalese sole and sea bass, and changes in concentrations of plasma variables after acute stress in rainbow trout (sampled after chasing for 15 min in tanks) and gilthead sea bream (sampled 20 min after air exposure for 3 min). Values are means ± S.E. of six Senegalese sole and sea bass, eight gilthead sea bream or 10 rainbow trout per group

		Plasma variables		
Species	Treatment	Cortisol (ng ml^{-1})	Glucose (μmol ml^{-1})	Lactate (μmol ml^{-1})
Rainbow trout	Control	5·65 ± 0·21	3·51 ± 0·18	1·21 ± 0·25
	Chasing	24·2 ± 0·84*	4·63 ± 0·23*	2·91 ± 0·27*
Gilthead sea bream	Control	5·56 ± 2·46	3·46 ± 0·31	3·55 ± 0·14
	Air exposure	144·6 ± 13·46*	6·02 ± 0·71*	5·21 ± 0·20*
Senegalese sole	Control	8·91 ± 0·44	3·32 ± 0·27	2·54 ± 0·64
Sea bass	Control	26·3 ± 1·19	3·78 ± 0·43	1·89 ± 0·66

*, significantly different from control group ($P < 0·05$).

being higher at 70 kg m^{-3} than in the other densities used. Plasma lactate concentration also displayed a relationship with stocking density showing a significant decrease in parallel with the increase in stocking density.

DISCUSSION

Specificity and validity of this ELISA system was demonstrated by three conventional ways such as: 1) the antiserum was tested for cross-reactivity with other steroids (not showing any significant bind with these hormones), 2) parallelism between serially diluted plasma of four different species (gilthead sea bream, Senegalese sole, sea bass and rainbow trout) with cortisol standards was observed and 3) this ELISA system was physiologically validated using experimental approaches (chasing, air exposure and stocking density) known to produce rises in plasma cortisol concentration in teleosts (Barton & Iwama, 1991; Wendelaar Bonga, 1997).

TABLE II. Plasma concentrations of cortisol, glucose and lactate in gilthead sea bream kept for 14 days under different stocking density conditions. Values are means ± S.E. of eight fish. Different lower case letters indicate significant differences ($P < 0·05$) among different stocking densities

	Stocking density (kg m^{-3})			
Plasma parameters	4	15	30	70
Cortisol (ng ml^{-1})	5·19 ± 1·47 a	6·70 ± 2·04 a	13·14 ± 1·67 b	27·30 ± 1·98 c
Glucose (μmol ml^{-1})	3·31 ± 0·11 a	3·52 ± 0·13 a	3·68 ± 0·14 ab	3·91 ± 0·12 b
Lactate (μmol ml^{-1})	4·21 ± 0·15 a	3·12 ± 0·12 b	2·45 ± 0·71 c	1·74 ± 0·09 d

The anti-cortisol antibody used in this ELISA showed low cross-reactivity with other steroids known to be present in fish plasma. An important advantage of this characteristic is that direct plasma can be used in the assay which eliminates time-consuming extraction and separation procedures required by some assays to eliminate cross-reactivity. In addition, parallelisms between the standard curve and plasma extract curves show that standard cortisol is recognized by its respective antibody in the same way as cortisol in plasma extracts.

There are several direct ELISA-based assay systems developed to measure plasma cortisol in teleosts (Barry *et al*., 1993; Nielsen *et al*., 1994; Carey & McCormick, 1998; Kelly & Woo, 1999). This is, as far as is known, the first indirect ELISA-based assay system developed to measure plasma cortisol in teleosts. The assay described here is specific for cortisol inasmuch as no significant cross-reactivity was found for other steroids within the working range of the ELISA. Furthermore, the recovery and plasma dilution series experiments indicated that spiked plasma samples and competitive displacement by non-spiked, serially diluted, plasma samples yielded a recovery of 98% and parallelism with the cortisol standard curve, respectively. The recovery observed is comparable to that observed in other assay systems either using RIA (Molinero & González, 1995) or ELISA (Barry *et al*., 1993). These results suggest the presence of little interference of plasma components in this assay system.

The reproducibility of the system (interassay CV <6%) is better than other ELISA systems described for fish plasma with values of *c*. 11% (Barry *et al*., 1993), 7·8% (Nielsen *et al*., 1994) and 8·8% (Carey & McCormick, 1998). As for precision of the system, (intra-assay CV), this was comparable with those of other ELISA systems (<3, 6·9 and 5·5%, in Barry *et al*., 1993; Nielsen *et al*., 1994 and Carey & McCormick, 1998, respectively). Both reproducibility and precision were also similar to those obtained in RIA-based systems (Laidley & Leatherland, 1988; Staurnes *et al*., 1994; Molinero & González, 1995; Arends *et al*., 1999; Barton, 2000; Marino *et al*., 2001).

The sensitivity of the present ELISA system (*c*. 0·3 ng ml^{-1}) is not as good as that of RIA based systems (Laidley & Leatherland, 1988; Staurnes *et al*., 1994; Molinero & González, 1995; Arends *et al*., 1999; Barton, 2000; Marino *et al*., 2001) though comparable to figures obtained in other ELISA based systems such as 0·1 ng ml^{-1} (Barry *et al*., 1993) or 0·3 ng ml^{-1} (Carey & McCormick, 1998), and better than in other ELISA systems such as 2·2 ng ml^{-1} (Nielsen *et al*., 1994). This make the assay less sensitive than RIA though nevertheless still adequate and robust for studying cortisol concentration in fishes.

Thus, the ELISA was accurate from *c*. 0·3 to 3000 ng ml^{-1} (using the protocol of 50 µl plasma samples diluted 1 : 40 for gilthead sea bream, rainbow trout and sea bass, or 1 : 50 for Senegalese sole). This covers the physiological range of cortisol concentration measured in most teleosts studied to date (Barton & Iwama, 1991; Mommsen *et al*., 1999). In addition, and because only small volumes of plasma are required, cortisol measurements can be made in very small fishes or in repeated plasma samples from the same fishes where small volume of plasma is available.

The cortisol values measured in non-stressed rainbow trout in the present experiment were *c*. 5 ng ml^{-1}, which is similar to the values already reported by other authors in the same species using different RIA or ELISA procedures

(Vijayan & Moon, 1992; Pottinger & Moran, 1993; Barry *et al.*, 1993; Nielsen *et al.*, 1994; Gamperl *et al.*, 1994; Barton, 2000; Sloman *et al.*, 2001). Cortisol concentrations measured in non-stressed gilthead sea bream were also similar to those reported previously for this specie in the literature (Molinero & González, 1995; Arends *et al.*, 1999; Montero *et al.*, 1999; Rotllant *et al.*, 2000; 2001; Laiz-Carrión *et al.*, 2002, 2003). A similar trend occurs in sea bass (Roche *et al.*, 1989; Cerdá-Reverter *et al.*, 1998; Pichavant *et al.*, 2001; Marino *et al.*, 2001; Rotllant *et al.*, 2003). As far as is known there are no references in literature regarding plasma cortisol concentration in Senegalese sole. Baseline concentration obtained in this species using the ELISA system, however, agree with data previously reported for other Pleuronectiformes (Waring *et al.*, 1992; Audet *et al.*, 1993; Sulikowski & Howell, 2003). The coincidence between baseline concentration measured in different species and those values already reported in literature for the same species give further support to the validity of the technique used.

A further checking to physiologically validate the ELISA was submitting fishes to different types of stressors. In the first experiment, freshwater-adapted rainbow trout were submitted to chasing in tanks for 15 min. The stressed fish displayed a significant increase in plasma cortisol similar to that already reported in different short-term stress situations in this species such as chasing (Nielsen *et al.*, 1994; Blom *et al.*, 2000), handling (Vijayan & Moon, 1992; Carey & McCormick, 1998; Barton, 2000), struggling (Barton & Schreck, 1987) and confinement (Pottinger & Moran, 1993; Biron & Benfey, 1994). The increased concentrations of plasma glucose and lactate probably reflect the action of cortisol and catecholamines elicited by stress, which agree with the reported effect of increased cortisol concentration on those metabolites (Mommsen *et al.*, 1999), and the known increase of those metabolites under stress situations (Barton & Iwama, 1991; Wendelaar Bonga, 1997).

In subsequent experiments, seawater-adapted gilthead sea bream were submitted to two different types of stressors: 1) a short-term stressor like air-exposure for 3 min and 2) a long-term stressor such as high stocking density in culture for 14 days. Air-exposure caused a sharp increase in plasma cortisol concentration reflecting the strong activation of stress system by this kind of treatment. This increase in cortisol concentration is similar to that observed in the same species submitted to identical stressor (Arends *et al.*, 1999) or to other short-term stressors such as handling (Rotllant *et al.*, 2001). The raised cortisol concentration also coincided with increased plasma glucose and lactate concentration and is in agreement with similar previous studies in the same species (Arends *et al.*, 1999). High stocking density is usually associated with marked changes in secretion rates of corticosteroids (Barton & Iwama, 1991; Wendelaar Bonga 1997). Thus, a possible increase in plasma cortisol concentration of gilthead sea bream submitted to increased stocking density conditions was assessed. The long-term stress effect of high stocking density also induced an increase in plasma cortisol concentration in fish maintained in densities from 15 kg m^{-3} onwards. Similar increases in parallel with increased stocking density have been observed in gilthead sea bream (Arends *et al.*, 1999; Montero *et al.*, 1999; Rotllant *et al.*, 2001) and other species (Vijayan *et al.*, 1990; Gamperl *et al.*, 1994). In addition, an increase in plasma glucose and a decrease in plasma lactate

are observed, which agree in general with changes expected after increased cortisol concentration in fishes (Mommsen *et al*., 1999; Laiz-Carrión *et al*., 2002, 2003) and after culture under high stocking density conditions (Vijayan *et al*., 1990).

Altogether, the different stress experiments performed with rainbow trout and gilthead sea bream provide further physiological validation for the indirect ELISA used since they gave not only increased concentrations of plasma cortisol (as expected from the treatments used) but also that the values were in the range of those previously assessed by other authors under similar experimental conditions.

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