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Inhibition of HPI axis response to stress in gilthead sea bream (*Sparus aurata*) with physiological plasma levels of cortisol

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Abstract

This study investigates the effect of corticosteroid (cortisol) administration on the stress response of the gilthead sea bream *Sparus aurata* subjected to a 48 h confinement. The effect of (*in-vitro* and *in-vivo*) cortisol administration on the *in-vitro* ACTH sensitivity of the interrenal tissue; the plasma levels and tissue concentration of cortisol; and the plasma levels of ACTH, α-MSH, β-endorphin and glucose were determined. Confinement caused a transient and concomitant increase in plasma cortisol and ACTH levels. However, in cortisol-fed fish the plasma ACTH levels were lower, indicating a suppresion of the ACTH release from the corticotropes by cortisol. In contrast to the activation of the corticotropes, the levels of plasma melanotrope derived peptides were not affected. In spite of the fact that interrenal cells of cortisol-fed gilthead sea bream released less cortisol than controls, the interrenal sensitivity to ACTH was not affected by *in-vivo* and *in-vitro* cortisol administration. This suggests that the interrenal sensitivity to ACTH in stressed (confinement) sea bream is probably not regulated by α-MSH, N-ac-β-END, or by cortisol. Thus, in gilthead sea bream the interrenal sensitivity to ACTH could be regulated at the hypothalamus and/or pituitary and communicated via circulating ACTH levels.

Abbreviations: ACTH – Adenocorticotropin hormone; α-MSH – α-melanocyte-stimulating hormone; N-ac-β-END – N-acetylated-β-endorphin; CRH – Corticotropin-releasing hormone; HPI – hypothalamus-pituitary-interrenal axis; POMC – Pro-opiomelanocortin; NPO – Preoptic nucleus; HK – Headkidney.

Introduction

Cortisol is the principal corticosteroid secreted by the interrenal cells located in the headkidney of the teleost fish (Balm et al. 1989; Patiño et al. 1987; Sangalang et al. 1972). The endocrine regulation of cortisol secretion in teleost fish is complex. Atrial natiuretic factor (Arnold-Reed 1991), angiotensin (Henderson et al. 1976), growth hormone (Ball et al. 1976), thyroxin (Young and Li 1988), arginin vasotocin (Schreck et al. 1981), catecholamines (Kloas et al. 1994) and other factors including interleukin-like factors of the immune system, may have corticotropic actions (Balm et al. 1993). However, the effects of these factors are probably no more than the modulation of the corticotropic action of pro-opiomelanocortin (POMC) derived peptides originating from the pituitary gland. ACTH (adrenocorticotropic hormone) has been considered the major factor controlling the cortisol synthesis and release of which in turn is regulated by the corticotropic releasing hormone (CRH) from hypothalamus (Donaldson 1981). In addition, alpha melanocyte-stimulating hormone (α-MSH) (Lamers et al. 1992) and N-terminally acetylated endorphin

(N-ac-β-END) (Balm et al. 1995) may also stimulate cortisol release.

Although there is evidence of the presence of intracellular glucocorticoid receptors in the brain of teleost (Lee et al. 1992; Knoebl et al. 1996), there is little information on the precise distribution and regulation of these receptors. Immunohistochemistry studies on trout (Tujague et al. 1998) revealed glucocorticod receptor-immunoreactive cells in the proximal pars distalis of the pituitary gland and in the preoptic nucleus (NPO) of the diencephalon. NPO is known to express arginine vasopressin and CRH and it is a major target for corticosteroids (Fuxe et al. 1985). This suggests that CRH-producing neurons are also likely to be regulated by corticosteroids. Moreover, cortisol has been shown to induce self-suppression by negative feed-back of its secretion directly at different levels: (i) the interrenal tissue (Bradford et al. 1992); (ii) the ventrodorsal hypothalamus; suppressing the synthesis and/or release of CRH (Fryer and Peter 1977); and/or (iii) the pituitary, inhibitting ACTH release (Fryer et al. 1984). Thus long-loop and ultra-short-loop feedback regulatory mechanisms have been documented in fish. However, the priority of the feeback events at the levels of hypothalamus, pituitary and interrenal tissue remains unsolved.

Besides its feedback actions on the corticotropic axis, stress and cortisol affects other vital physiological functions of fish, such as reproduction (Carragher 1990; Foo and Lam 1993), osmoregulation (Mancera et al. 1994; Reddeing et al. 1991), growth (Barton et al. 1987), and immunity (Espelid et al. 1996; Nagae 1994; Rotllant et al. 1997; Weyts et al. 1998). Thus cortisol has a key role in regulating many important physiological functions in teleosts.

To further study the role of these feedback events, we administered *in vivo* and *in vitro* a high physiological dose cortisol. We also confined the fish to evoke an activation of the Hypothalamus-pituitary-interrenal (HPI) axis for investigating the influences of stress on interrenal ACTH sensitivity and on factors regulating this parameter. The aim of these experiments was to discriminate between cortisol and ACTH as possible mediators of the stress effects on the parameters measured. In addition, (a) the headkidneys of experimental fish were superfused *in vitro*, paying particular attention to ACTH responsiveness, and (b) plasma levels of cortisol, ACTH, α-MSH, N-ac-β-END, and glucose were determined.

Material and methods

Animals

Sexually immature gilthead sea bream (*Sparus aurata* L.), weighing 75 to 150 g, were obtained from an experimental fish culture center (El Toruño, PEMARES, El Puerto de Santa María, Cádiz). During the experiments (May–July, 1998), the fish were kept under environmental conditions of photoperiod (14L:10D) and water temperature (22–24° C). Three groups of fish were used in different experiments. They were kept in well-aerated 5000 l stock-tanks at a density of 4 kg m^{-3}. The water was continuously refreshed (250 l h^{-1}) and supplied with air through air stones. Fish were fed twice a day (9 am and 6 pm) with 1% body weight commercial dry pellets (DIBAQ-DIPROTEG S.A., Segovia, Spain).

Experimental protocol

Oral corticosteroid administration: Time-course response on plasma cortisol concentration.
It was intended to study the time-course cortisol response after oral exogenous cortisol administration, and the effect of cortisol feeding. Hence, 88 fish (8 fish per group and one group for each time-point) were transferred from their stock tank to grey cylindrical experimental tanks (V = 500 *l*; diameter tank: 0.85 m), containing a plastified iron wire-net cage with a total volume of 250 l (inner diameter cage: 0.60 m) to obtain a fish density of 4 kg m^{-3}. The fish were allowed to acclimate to the experimental tank for 6 days. Fish were fed a cortisol meal (1% body weight; 400 mg cortisol kg^{-1} food). The meal was given just before the first sampling (0 h). The fish were then sampled immediately (0 h) and after 1, 3, 6, 11, and 24 h. The experimental diet was prepared by dissolving hemisuccinate-cortisol (Sigma, St. Louis, USA) in 100% ethanol and then spraying the resultant solution onto the surface of the pellets to produce a concentration of 400 mg cortisol kg^{-1} food (Pickering 1983). The control diet was prepared by using an equivalent amount of ethanol only. Feed pellets air-dried for 1 h with occasional stirring to evaporate the ethanol, and then refrozen. No mortality was observed in any group during the experiments. To minimize confounding effects of tank-related influences, control and experimental groups were sampled from randomly chosen tanks at randomly chosen time-points during each experiment.

48 h confinement stress

To study the effect of transient elevation in plasma cortisol levels on HPI response to 48 h confinement, fish were pretreated 36 h and 6 h with cortisol before confinement, delivered via the food following the above protocol. Confinement consisted of lifting the wire-net cage in the tank (water depth about 10 cm) to increase the stocking density from 4 to 70 kg m^{-3}. Previous studies have shown that this system induces an activation of the HPI axis in gilthead sea bream with an increase in plasma cortisol levels (Arends et al. 1999a). Fish ($n = 8$ per group) were sampled at 0 (before confinement) and at 1, 4 , 24 and 48 h during confinement. Fish were not fed during confinement protocol.

In-vitro *superfusions of sea bream headkidneys*

Headkidneys were quickly dissected after sampling. The tissues were placed in superfusion chambers and superfused with a buffered Hepes Ringer solution (Hepes 15 mM; pH 7.38) containing NaCl (171 mM), KCl (2 mM), $CaCl_2 \cdot 2H_2O$ (2 mM), 0.25% (w/v) glucose and 0.03% (w/v) bovine serum albumin and adjusted to sea bream osmolality (369 mOsm kg^{-1}) (Pages et al. 1995). This medium was pumped through the superfusion chambers at a rate of 75 μl min^{-1} by means of a multichannel peristaltic pump (Gilson). To study the *in-vitro* effect of exogenous cortisol, this medium was either supplemented with hemisuccinate-cortisol (Sigma, St. Louis, USA) at different concentrations or not supplemented. This system was temperature controlled at 20 °C. Previous results indicated that cortisol reached at a stable baseline level after 3 h of superfusion (Rotllant et al. non-published data). Therefore, after 3 h, tissue was stimulated with ACTH at a concentration of 5 nM ($hACTH_{1-39}$, Sigma) for 20 min. This concentration appeared submaximal (a). For each fish the maximum cortisol release due to ACTH stimulation was compared with the baseline release in order to obtain the stimulation factor of ACTH, defined as (maximum release − baseline release)/(baseline release).

Sampling

All fish belonging to the same sampling group were rapidly captured and anaesthetised with 2-phenoxyethanol (1:2000; Sigma). Fish were bled from caudal vessels and the blood was treated with EDTA/Aprotinin (1.5 mg/250 KU/ml blood; Sigma). Fish were anaesthetized in about 20–30 s and the whole procedure took place in less than 3 min in order to avoid changes in the variables measured due to sampling. Plasma aliquots were separated and frozen (−20 °C). Homogenates of headkidneys were prepared with 0.01N HCl and kept frozen (−20 °C) until analysis was performed.

Analytical procedures

Cortisol was measured by well-established and validated radioimmunoassay (RIA) for sea bream (Molinero and Gonzalez 1995). The RIA for α-MSH was based on an antibody described by Vaudry et al. (1978), and was used in a final dilution of 1:60 000. The cross-reactivity of this antiserum with des-acetylated, mono-acetylated and di-acetylated α-MSH is 100%. Immunocomplexes were precipitated with 15% (w/v) polyethylene glycol and 2.4% (w/v) ovalbumin as described previously (van Zoest et al. 1989). ACTH was measured in a RIA described by Balm et al. (1994) for tilapia, using an antibody raised in rabbit against human $ACTH_{1-24}$ (Dores et al. 1993). Immunocomplexes were collected by precipitation with a sheep-anti-rabbit second antibody and 7.5% (w/v) polyethylene glycol. Pituitary homogenates of sea bream prepared in 0.01 m HCl and diluted in RIA assay buffer displaced radiolabeled ACTH from the antibody in parallel with dilutions of the standards used. Cross-reactivity with α-MSH was negligible. For the RIA of β-endorphins an antiserum recognizing N-terminally acetylated endorphins was used (Takahashi et al. 1984). The antiserum has full cross-reactivity with acetylated forms of mammalian β-endorphins, while cross-reactivity with non-acetylated endorphins is less than 0.1% (Dores et al. 1991). For RIA, the antibody was used in a final dilution of 1:100 000. Immunocomplexes were precipitated with 15% (w/v) polyethylene glycol and 2.4% (w/v) ovalbumin. Plasma glucose was measured using a commercial kit from Sigma (Iwama et al. 1989).

Statistics

Results are given as mean ± standard error of the mean (SEM) for each group ($n = 8$). First, one-way analysis of variance (ANOVA) was applied followed by the Student–Newman–Keuls (SNK) test to check differences between specific groups. When non-homogeneous variances were obtained, a logarithmic transformation was performed before statistical

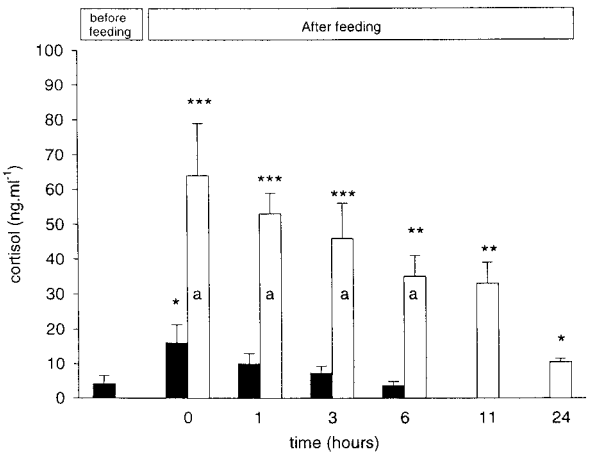

Figure 1. Changes in the plasma cortisol concentration of gilthead sea bream (*S. aurata*), following oral administration of the steroid as a single meal (400 mg cortisol kg^{-1}; 1% body weight). Non-cortisol-fed fish (dark bars) and cortisol-treated fish (light bars). (a) denote significant differences between both groups at that particular time point and (*) denote significant differences with the pre-feeding control. *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$). Values are mean±SEM ($n = 8$).

analyse was applied. Where logarithmic transformation did not result in homogeneous variances the test of Kruskal–Wallis (KW) was performed followed by the Mann–Whitney (U) for a posteriori test (U-test; $p < 0.05$). The level for accepted statistical significance was $p < 0.05$. Significant difference with $t = 0$ within that treatment is indicated by '*', significant difference between control and cortisol treated fish for that hour is indicated by 'a' ($^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$).

Results

Oral corticosteroid administration: time-course response on plasma cortisol concentration

The effects of oral administration of cortisol on plasma cortisol levels are illustrated in Figure 1. The cortisol dose (400 mg kg food^{-1}; 1% body weight) prior to sampling caused a significant elevation of plasma cortisol levels compared to the fish fed with control food. Thus, plasma cortisol levels increased from 16.0 ± 5 at $t = 0$ *h* in control fish to 64.0 ± 15 ng ml^{-1} in cortisol-fed fish ($p < 0.05$). After 6 h, fish fed with control food showed plasma cortisol levels similar to the initial control levels (3.6 ± 1.2 vs 4.2 ± 2.3 ng ml^{-1} respectively). However, the fish fed with exogenous cortisol still showed high plasma cortisol levels compared to controls (35.0 ± 6 vs 4.0 ± 1 ng ml^{-1}, respectively). No control data at 11 h and 24 h were recorded.

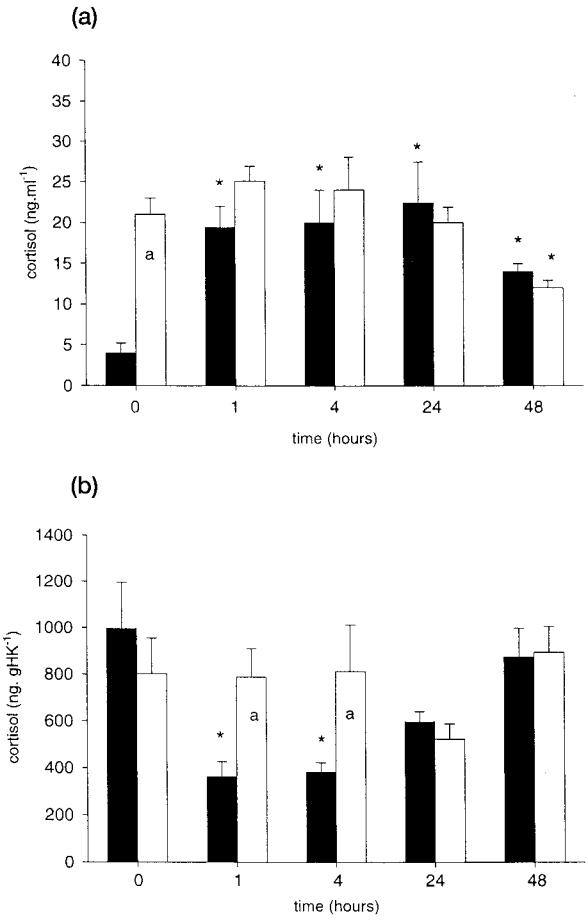

Figure 2. Changes in plasma (a) and interrenal tissue (b) cortisol levels in non-cortisol-fed (dark bars) and cortisol-treated (light bars) sea bream subjected to 48 h confinement. Key as for Figure 1.

48 h confinement stress

Confinement caused a transient increase in plasma cortisol levels in non-fed cortisol fish (Figure 2a). Plasma cortisol levels rose from baseline levels of 4.0 ± 1.2 to 19.4 ± 1.2 ng ml^{-1} ($p < 0.05$). After 48 h, plasma cortisol levels were still higher than the control values ($p < 0.05$). Confinement also produced a transient reduction of the tissue cortisol levels (Figure 2b). Within 1h, interrenal cortisol contents decreased from control values of 992 ± 200 to 362 ± 35 ng gHK^{-1} ($p < 0.05$). After 24 h, a recovery was observed as levels were similar to those of control. Similar to cortisol, plasma ACTH displayed a transient increase

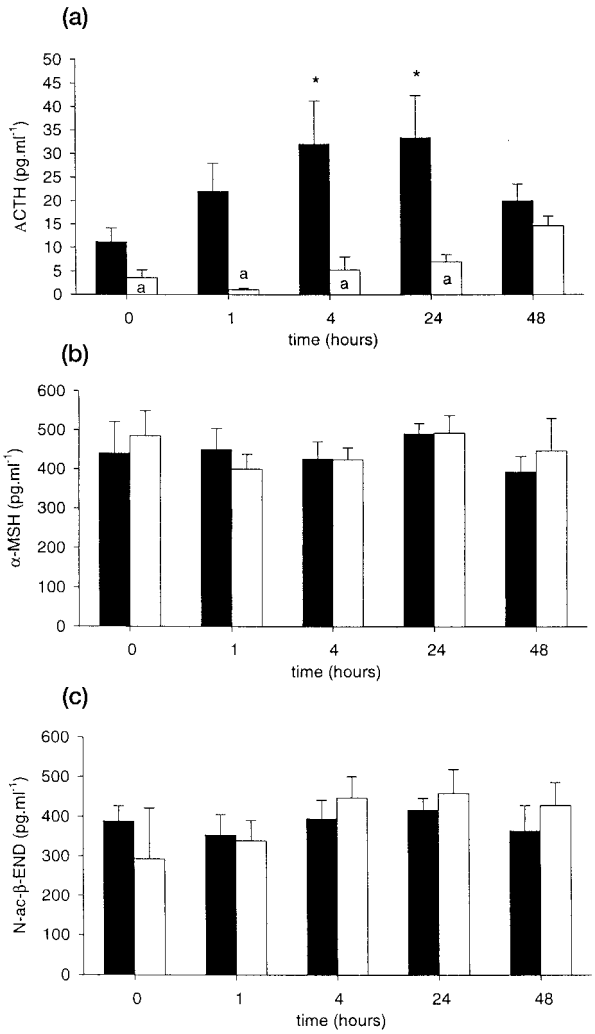

Figure 3. Changes in plasma ACTH (a), α-MSH (b) and N-ac-β-END (c) levels in non-cortisol-fed (dark bars) and cortisol-treated (light bars) sea bream subjected to 48 h confinement. Key as for Figure 1.

(Figure 3a). Thus, after 4 h confinement, plasma ACTH increased from control values of 13.4 ± 2.6 to 32 ± 9.2 pg ml^{-1} ($p < 0.05$). However, after 48 h the levels of plasma ACTH in confined fish were similar to the controls. Regarding the cortisol fed fish, a significant increase of cortisol (Figure 2a) was apparent at the beginning of the experiment and the same high levels were maintained until the end of the experiment. However, ACTH levels were significantly lower than for control fish ($p < 0.05$), reaching control values after 48 h (Figure 3a).

In contrast to ACTH, confinement had no effect on plasma levels of α-MSH or β-endorphin in control

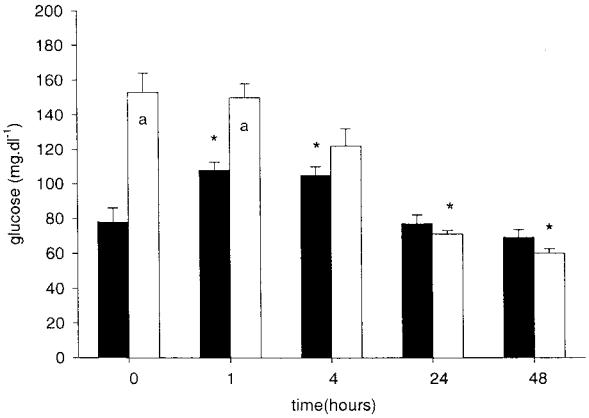

Figure 4. Changes in plasma glucose levels in non-cortisol-fed (dark bars) and cortisol-treated (light bars) sea bream subjected to 48 h confinement. Key as for Figure 1.

and cortisol fed fish (Figure 3b,c). Forty-eight hours confinement slightly increased plasma glucose after 1 h ($p < 0.05$) in control fish (Figure 4). Cortisol-fed fish had significantly increased glucose levels at the begining of the experiment and at 1 h of confinement ($p < 0.05$) compared to control fish. Control fish showed an expected increase of plasma glucose levels at 1 h and 4 h of confinement (Figure 4)

In-vitro *superfusion of sea bream headkidneys*

Table 1 shows the steroidogenic response (expressed as ACTH stimulation factor) of headkidneys from sea bream *in-vitro* superfused with a medium either supplemented with cortisol (20 and 200 ng ml^{-1}) or not supplemented and both stimulated with 5 nM of $hACTH_{1-39}$ for 20 min. The ACTH sensitivity of interrenal tissue of non-cortisol-fed sea bream (expressed as ACTH stimulation factor) was 79 ± 10 in the control and was not altered by cortisol administration in-vitro at the two different concentration tested. In cortisol-fed fish, again the ACTH stimulation factor was not different from non-cortisol-fed sea bream.

Figure 5a shows that a 20 min *in-vitro* pulse of ACTH elicited responses with similar kinetics in both control and cortisol-fed fish. The area under the curve was similar in control and in cortisol-fed fish prior to confinement (366 ± 41 vs 378 ± 33, respectively). However, confinement affected differently the cortisol *in-vitro* release in each group. Figure 5b–d. summarises the *in-vitro* superfusion data for all sample points. The two groups released different amounts of cortisol in the absence of ACTH (Figure 5b). The initial unstimulated cortisol release was lower in cortisol-fed fish

Table 1. Sensitivities of interrenal tissues of control to ACTH (expressed as ACTH stimulation factor) under cortisol administration

	Plasma cortisol (ng mL^{-1})	Medium cortisol (ng mL^{-1})	ACTH stimulation factor
Control	4.2 ± 2	–	79 ± 10
Cortisol *in-vitro*	3.4 ± 1	20	62 ± 14
		200	73 ± 11
Cortisol fed	$21.2 \pm 2^*$	–	81 ± 12

The effect of cortisol *in vivo* and *in vitro* on the sensitivity of cortisol producing tissue to ACTH. Values are mean±sem. $^*(p < 0.05)$.

($p < 0.05$) at 0 h and 1 h of confinement, reaching the control values by 24 h of confinement. Confinement let to an increase of the baseline unstimulated cortisol release at 24 h and 48 h ($p < 0.001$) in non-fed-cortisol fish compared to control (0 h) values. However, in cortisol treated fish confinement did not affect the baseline cortisol release during all the experimental period (Figure 5c). The maximal ACTH response, expressed as the stimulation factor over prepulse release, was significantly lower only in non-cortisol-fed 24 h ($p < 0.001$) and 48 h ($p < 0.001$) confined group compared with the respective control (Figure 5d). However, in confined cortisol-fed fish, the ACTH stimulation factor was not affected by confinement, showing non-significant differences in all the confined period compared with values prior to confinement (Figure 5d).

Discussion

Oral cortisol administration

In this type of study it is important that the levels of administered hormone fall within the physiological range normally experienced by the animals. The low cortisol levels for control fish after 6 h of the second feeding (<5 ng ml^{-1}) are similar to those measured in previous occasions (Tort et al. 1996; Rotllant et al. non-published data) in gilthead sea bream. The significant plasma cortisol increase following administration of exogenous cortisol confirms that the oral cortisol administration is suitable for studies in which elevated levels of cortisol are wanted, but at the same time, it is imperative that handling stress was avoided (Pickering and Duston 1983). After 6 h of the second cortisol feeding, the peak of cortisol ($\sim$35 ng ml^{-1}) is well within the physiological range for sea bream and it is similar to that observed when sea bream are stressed by short-term confinement (see Figure 2). Thus, the experimental administration of cortisol to the sea bream in the present study produces changes in plasma cortisol levels which approximate those that occur in sea bream subjected to short-term confinement. This experimental design allowed us to compare between the effects of elevated cortisol levels in the presence of low circulating ACTH levels (cortisol-fed fish), and the effects of elevated cortisol levels in the presence of elevated ACTH levels (confined fish) in order to discriminate between cortisol and ACTH as possible mediators of the stress effects on the parameters analysed.

48 h confinement stress

A transient stress response (both at the pituitary and interrenal level) was observed in control gilthead sea bream under confinement conditions, as previously shown by several authors for gilthead sea bream (Arends et al. 1999a; Rotllant et al. non-published data). Thus, the fact that sea bream subjected to confinement for 1h. showed a coincident increase of cortisol and ACTH levels (Figures 2 and 3) and the decline of ACTH levels in confined fish precedes the drop in cortisol, suggest that under this confinement conditions, ACTH is the major factor regulating cortisol levels. These data suggest that the habituation of stress response in sea bream is, to a substantial extent, regulated at the hypothalamic-pituitary levels by a decline in ACTH levels, as found in mammals (Sapolsky et al. 1984; Dallman 1993). Furthermore, our data demonstrate that fish treated with exogenous cortisol prior to confinement have lower plasma ACTH levels than control fish. This confirms the work of Arends et al. (1999b) and shows that cortisol acts in sea bream by suppressing the release of ACTH from the corticotropes. Data also support other observations on dexamethasone-treated fish (Pickering et al. 1987;

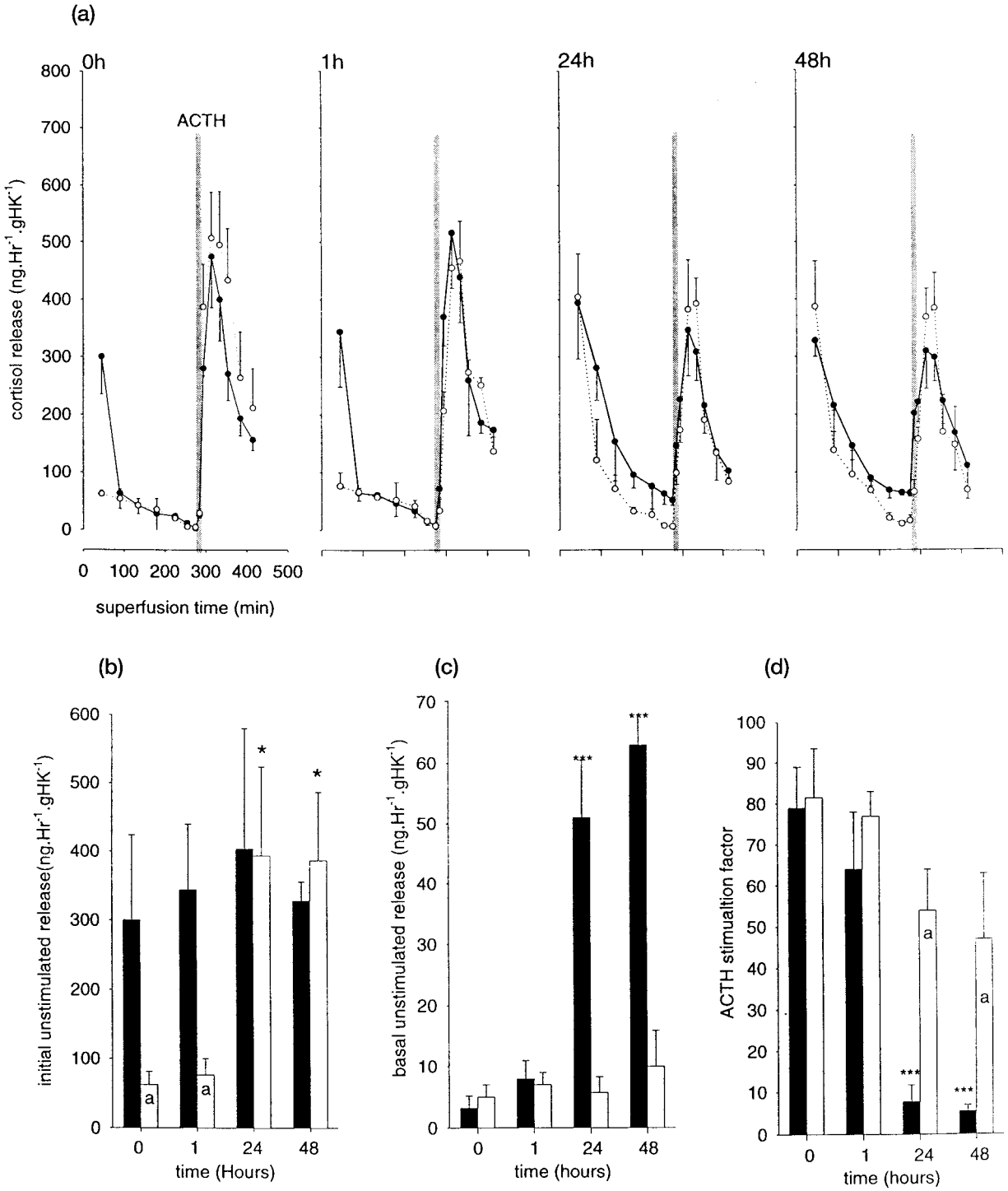

Figure 5. Comparison and analysis of the effects of 48 h confinement and cortisol treatment on *in-vitro* cortisol release by headkidneys. Tissue was pulsed with 5 nM ACTH$^{(1-39)}$ for 20 min. (a) Kinetics of the *in-vitro* cortisol release by interrenal tissue in response to a pulse of ACTH. Non-cortisol-fed fish (dark line) and cortisol-treated fish (dotted line). Initial release (b) was measured in the first 45 min fraction collected *in vitro*. Baseline release (c) was the average from two fractions collected at $t = 255$ and 275 min. The ACTH stimulation factor (d) was calculated from (maximal release – prepulse release)/prepulse release. Non-cortisol-fed fish (dark bars) and cortisol-treated fish (light bars). Key as for Figure 1.

Sumpter and Donaldson 1986) where dexamethasone treatment significantly suppressed basal ACTH levels in the brown trout (down to 10 pg ml^{-1}). In contrast to the activation of the corticotropes, it is demonstrated that confinement did not affect the melanotropes. Our data show that confinement did not induce significant changes in plasma α-MSH and N-ac-β-END levels, supporting previous observations on gilthead sea bream under handling conditions (Rotllant et al. non-published data). However, it has been shown that other stressors such as crowding (fish density, 30 kg m^{-3}) rapidly affect the levels of both α-MSH and N-ac-β-END in this species (Rotllant et al. non-published data). Thus, the particular characteristics of each type of stress determine which cell type or types became active, supporting pervious works by several authors (Malo-Michelle 1980; Sumpter et al. 1985; Balm et al. 1995). Furthermore, fish treated with exogenous cortisol prior to confinement did not show significant differences in plasma α-MSH and N-ac-β-END levels compared to the controls (Figure 3). Thus, cortisol does not inhibit the melanotropic cells in the pars intermedia. This is in agreement with previous findings in cortisol-injected sea bream (Arends et al. 1999b). Regarding the metabolic responses, our results support those obtained in a previous study (Arends et al. 1999b) reporting that the effect of cortisol injection in sea bream resulted in an increase in plasma glucose levels at the beginning of confinement due to a hyperglycaemic effect of cortisol. In non-cortisol-fed fish plasma glucose levels were significant elevated above control levels after 1h of confinement. This is likely due to enhanced glycogenolysis, as reported by Vijayan et al. (1997).

In-vitro *superfusion of sea bream headkidney: Interrenal sensitivity to ACTH*

The concentration of ACTH used in the present superfusion studies was selected because previous work (Rotllant et al. non-published data) reported it to be near the EC50 value in sea bream, and therefore this range of concentrations is a suitable one for assessing treatment-induced increases or decreases of interrenal ACTH sensitivity.

Regarding *in-vitro* effects, cortisol administration in the superfusion medium had no effect on the sensitivity of the interrenal cells to ACTH (expressed as ACTH stimulation factor; Table 1). Thus, the data suggest that sea bream interrenal cells are relatively insensitive to direct cortisol feedback, which may seem surprising in view of previous *in-vitro* results on salmon (Bradford et al. 1992). However, this discrepancy could be attributed to the use of the different *in-vitro* superfusion systems. In contrast to Bradford et al. (1992), that used a static superfusion system, we have used a dynamic system. This dynamic system offers several obvious advantages over the static superfusion of headkidneys. Perhaps the most important is that the continuous flow of medium prevents the build-up of high levels of cortisol. Furthermore, fish treated with exogenous cortisol, showed an ACTH response consisting of either a stimulation over basal release (as shown by the stimulation factor) or in absolute values (area under the curve) which are not significantly different from values of control fish (Figure 5). Our data on the effects of confinement indicate that modulation of the interrenal sensitivity to ACTH occurred in control confined fish, since the ACTH stimulation factor significantly declines within 24 h, supporting previous observations on confined trout and sea bream (Balm et al. 1995; Rotllant et al. non-published data). The reduction in interrenal ACTH sensitivity was observed prior to the decline in plasma ACTH levels, which does not occur until 48 h confinement. However, no effect of confinement on the ACTH interrenal sensitivity was evident in fish treated with exogenous cortisol prior to confinement, since the ACTH stimulation factor was similar at all sample points. Thus, in sea bream the interrenal ACTH sensitivity appears not to be predominantly regulated by cortisol. This is corroborated by results obtained by Balm et al. (1995) in rainbow trout (*Oncorhynchus mykiss*). Furthermore, our data on the effects of confinement on the basal unstimulated cortisol release show a positive relationship with plasma ACTH levels, consistent with data on coho salmon (*Oncorhynchus kisutch*) and trout (*Oncorhynchus mykiss)*, where rearing densities affect basal unstimulated release (Patiño et al. 1987; Balm et al. 1995). Thus, we support the work by Balm et al. (1995) postulating that the effect of confinement on the *in-vitro* basal cortisol output reflects the sustained effect of prolonged exposure to elevated plasma ACTH levels. Also, our data demonstrate that a direct effect of circulating cortisol levels on basal cortisol production rates *in-vitro* can be excluded, since cortisol treated fish subjected to confinement did not mimic the effect of confinement on basal cortisol production in control fish.

In summary, the present study indicates that oral cortisol administration inhibits the stress response of sea bream to confinement. However this inhibition is

only evident for corticotropes. The interrenal ACTH sensitivity in stressed sea bream is probably not regulated by α-MSH and N-ac-β-END nor by cortisol. Thus, in sea bream the ACTH interrenal sensitivity would be regulated by the hypothalamus and/or pituitary and communicated via circulating ACTH levels, supporting similar mechanisms identified in mammals (Cone and Mountjoy 1993; Penhoat et al. 1994).

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