Modulation of ACTH-induced cortisol release by polyunsaturated fatty acids in interrenal cells from gilthead seabream, *Sparus aurata*

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Abstract

Highly unsaturated fatty acids are essential components of cellular membranes of vertebrates and can modulate physiological processes, including membrane transport, receptor function and enzymatic activities. In gilthead sea bream, dietary deficiencies of essential fatty acids of marine fish raise the basal cortisol levels and alter the pattern of cortisol release after stress. The aim of the present study was to clarify the effect of different essential fatty acids on adrenocorticotropic hormone (ACTH)-induced cortisol production and release in fish, through *in vitro* studies of sea bream interrenal cells maintained in superfusion and incubated with different types of fatty acids and eicosanoid production inhibitors. Results showed the first evidence of the effect of certain fatty acids on cortisol production by ACTH-stimulated interrenal cells in fish. Both arachidonic acid (ARA) and particularly eicosapentaenoic acid (EPA) promoted cortisol production in sea bream interrenal cells. Moreover, incubation with indometacin (INDO) reduced the increased cortisol production induced by EPA and ARA, suggesting mediation by their cyclooxygenase-derived products. Docosahexaenoic acid stimulated cortisol production to a lesser extent than that caused by EPA or ARA, but the inhibitory effect of INDO was not as marked as it was for the other fatty acids. In contrast, supplementation with dihomogammalinoleic acid reduced cortisol production, denoting the inhibitor effect of this fatty acid in cortisol secretion.

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Introduction

Highly unsaturated fatty acids with 20 or more carbon atoms and three or more double bonds (HUFA) are essential components of cellular membranes and can modulate physiological processes, including membrane transport, receptor function and enzymatic activities. Hence, dietary fatty acids have been shown to have marked effects on a variety of immunological and haemostatic parameters (Balfry *et al.* 2001, Montero *et al.* 2001). HUFA possess a wide range of cellular functions. One of the most important functions is to supply precursors for the synthesis of eicosanoids, which are produced in response to various extracellular stimuli by two main types of dioxygenase enzymes: cyclooxygenases (COX) and lipoxygenases (Horrobin 1983). Following cell stimulation, both arachidonic acid (ARA; 20:4n-6) and eicosapentaenoic acid (EPA; 20:5n-3) are released from the membrane by the action of phospholipase A_2. Later these fatty acids are transformed by a range of lipoxygenases and cyclooxygenases to yield prostaglandins (PG), leukotrienes, lipoxins and other compounds, which can modulate several immune functions (Uhing *et al.* 1990).

Eicosanoids have been found in a large range of freshwater and marine fish (Matsumoto *et al.* 1989, Mustafa & Srivastava 1989) and in many tissues (Henderson & Tocher 1987, Bell *et al.* 1994*a*, Tocher 1995). In fish, a preferred eicosanoid precursor for cyclooxygenase seems to be ARA (20:4n-6) (Tocher & Sargent 1987, Bell *et al.* 1994*a*, 1994*b*, 1998), but EPA (20:5n-3) and dihomo-γ-linolenic acid (DHGLA; 20:3n-6) are also important eicosanoid precursors which can modulate production and biological efficacy of ARA-derived eicosanoids (Horrobin 1983, Bell *et al.* 1994*a*, Ganga *et al.* 2005). In addition, the high content of docosahexaenoic acid (DHA; 22:6n-3) in cellular membranes affects eicosanoid production (Nablone *et al.* 1990). This fatty acid is also recognised as a precursor of certain biologically active trioxilated derivatives (German *et al.* 1983, Hong *et al.* 2005). Therefore, the supply of precursor polyunsaturated fatty acids with 18 or more carbon atoms and two or more double bonds (PUFA) for eicosanoid synthesis is directly related to the fatty acid composition of membrane phospholipids, which in turn is influenced by dietary PUFA intake and metabolism (Lands 1989).

In gilthead sea bream, dietary deficiencies on n-3 HUFA, essential fatty acids for marine fish (Izquierdo 1996), raised the basal plasma cortisol levels and altered the pattern of cortisol release after stress (Montero *et al.* 1998). Cortisol is a key corticosteroid hormone for homeostatic response to stress

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in all vertebrates, through its effects on metabolism and immune function (Hontela 1997, Wendelaar Bonga 1997) as well as the osmoregulation process (Wendelaar Bonga 1997). Thus, the increase in plasma cortisol levels is regarded as the most reliable method for differentiating between stressed and non-stressed fish (Thompson *et al.* 1993, Yin *et al.* 1995, Rotllant & Tort 1997). Moreover, feeding relatively low levels of n-3 HUFA, although not affecting growth and feed efficiency, significantly raised plasma cortisol levels (Montero *et al.* 2003).

However, the physiological mechanisms by which these HUFA regulate the hormone-induced plasma cortisol levels are not clear. In fish, several studies have suggested that ARA is involved in the release of cortisol, although the actual mechanisms have not been investigated (Gupta *et al.* 1985, Bessonart *et al.* 1999, Harel *et al.* 2001, Koven *et al.* 2003, Van Anholt *et al.* 2004). In mammals, certain studies suggest that PG play an important role in mediating the corticosteroidogenic action of adrenocorticotropic hormone (ACTH) (Kocsis *et al.* 1999), and thus the role of fatty acids in stress response seems to be mediated by the production of eicosanoids.

The present study aims to clarify the effect of different HUFA on ACTH-induced cortisol production and release by gilthead sea bream interrenal cells.

Material and Methods

Animals

Sexually immature gilthead sea bream (*Sparus aurata*) of body weight $54{\cdot}7 \pm 11{\cdot}2$ g supplied by a Spanish fish farm (Masnou, Barcelona, Spain) were kept for 2 weeks in two fibreglass tanks of 1000 l held in a semi-closed seawater circulation system equipped with physical and biological filters. Water temperature was maintained at 16–18 °C, the salinity at 35–40% and photoperiod at 12 h light:12 h darkness. Fish were fed once a day with a commercial feed until 24 h before the *in vitro* trials to avoid feed interference. A total number of 30 fish were employed in the experiments.

Superfusion trials

After 2 weeks of acclimatisation, fish were randomly taken from the tanks in less than 1 min, immediately anaesthetised with 2-phenoxyethanol (1:1000 v/v) and blood collected with a hypodermic syringe from the caudal vein to minimise the haemorrhage. Head kidney tissue was removed from two fish in each superfusion trial and cut into very small fragments in Hepes Ringer medium, which was used as the superfusion medium. Afterwards, head kidney homogenates were pooled and distributed in eight superfusion chambers (volume: 0·2 ml) in order to obtain a homogeneous aliquot from each of them. Tissues were superfused with a Hepes (pH 7·4) Ringer's solution containing 171 mM NaCl, 2 mM KCl, 2 mM $CaCl_2H_2O$, 0·25% (w/v) glucose and 0·03% (w/v) bovine serum albumin (Rotllant *et al.* 2001). The system was temperature-controlled at 15 °C and superfusion medium was pumped through the chamber at a rate of 75 µl/min by a Masterplex L/S^R multichannel peristaltic pump (Cole Parmer Instrument Co. Vernon Hills, IL, USA).

Trials were started after 3 h of superfusion when cortisol reached a stable baseline level (Rotllant *et al.* 2000*a*, 2000*b*) due to several factors such as the different dispersion of interrenal cells in the perfusion preparation, individual differences and the pre-stress level of each fish. After the stabilisation period of 3 h, tissues were subsequently incubated with different fatty acids. A series of preliminary tests were performed in quadruplicate, to determine the adequate fatty acid concentration (50, 150 or 300 µM) and incubation time (1 or 3 h) for any of the three fatty acids assayed (ARA, EPA and DHA). Best cortisol stimulation was found with fatty acid concentrations of 50 µM and an incubation time of 1 h (Table 1) and these conditions were used afterwards in all the research experiments. Both in these preliminary tests and in the research experiments, perfusion medium was supplemented with the corresponding concentration of different fatty acids ARA, EPA, DHA and DHGLA (diluted in less than 0·5% of ethanol/medium v/v) prior to tissue incubation. In a second series of experiments to clarify the action mechanisms of these fatty acids, tissues were incubated with a COX inhibitor indometacin (INDO) for 20 min at a concentration of 25 µM diluted in superfusion medium. After incubation with the fatty acids, the perfused tissues were stimulated with ACTH at a concentration of 5 nM $hACTH_{1–39}$ (Sigma) for 20 min. Subsequently, perfusion was maintained for another 170 min, fraction samples being collected every 20 min during this period. Cortisol stimulation factor was calculated by the comparison of maximum cortisol released after ACTH stimulation with baseline cortisol released (maximum release − baseline release)/(baseline release) (Rotllant *et al.* 2001). In all the series of experiments, each treatment was assayed in quadruplicate.

Cortisol measurements

Cortisol concentration in the perfused fluid was determined by RIA (Rotllant *et al.* 2001). The antibody used for the assay was purchased from Biolink, S.L. (Costa Mesa, CA, USA) in a

Table 1 Effect of two fatty acid concentrations (50 and 150 µM) and two incubation times (1 h and 3 h) for three polyunsaturated fatty acids on cortisol secretion stimulation factor

		1 h	3 h
Treatment			
Control		$14{\cdot}71 \pm 2{\cdot}41$	13·28
EPA	50 µM	$29{\cdot}63 \pm 2{\cdot}59$	$7{\cdot}79 \pm 3{\cdot}29$
	150 µM	$7{\cdot}79 \pm 3{\cdot}29$	–
ARA	50 µM	$22{\cdot}26 + 6{\cdot}29$	$11{\cdot}75 \pm 4{\cdot}16$
	150 µM	$12{\cdot}25 \pm 1{\cdot}86$	–
DHA	50 µM	$35{\cdot}72 \pm 9{\cdot}28$	$2{\cdot}60 \pm 1{\cdot}16$
	150 µM	$4{\cdot}47 \pm 0{\cdot}28$	–

final dilution of 1:6000. This antibody cross reactivity is 100% with cortisol, 11·40% with 21-desoxycorticosterone, 8·90% with 11-desoxycortisol and 1·60% with 17α-hydroxyprogesterone. The radioactivity was quantified using a liquid scintillation counter. Cortisol levels are given as ng/g/h.

Statistical analysis

Significance of difference ($P<0·05$) between dietary treatments was determined by ANOVA, followed by Duncan's multiple comparison test (Sokal & Rolf 1995). Analyses were performed using SPSS software (SPSS for Windows 11·5; SPSS Inc., Chicago, IL, USA).

Results

The different incubation times and fatty acid concentrations assayed showed that 1 h of incubation time and a concentration of 50 μM of fatty acid were the best conditions to obtain the highest effect of fatty acid on cortisol secretion stimulation factor (Table 1). As expected, after the stabilisation period of 3 h, cortisol values remained at basal levels for these fish species and no significant differences were found among basal values for the different superfused tissues (Fig. 1).

The effects of supplementation with different HUFA on cortisol secretion are illustrated in Fig. 1. The production of cortisol by interrenal cells was modified when the medium was supplemented with HUFA in comparison with the control. Addition of n-3 fatty acids, DHA and EPA induced a higher and earlier cortisol response to ACTH than the control without fatty acid incubation. Addition of n-6 fatty acids did not modify the time of cortisol response in comparison to the control, but induced a higher response. Cortisol response was higher when ARA, EPA or DHA was added to fatty acid and lower when DHGLA was used. Such response expressed as stimulation factor was significantly ($P<0·05$) higher with EPA (33·71±4·5 basal secretion) and ARA (28·7±4·47) incubation than control and DHGLA treatment groups (Fig. 2). With DHA incubation, no significant differences were found in the stimulation factor. By contrast, DHGLA showed the lowest ($P<0·05$) stimulation factor with an increase of only 8·95±2·17.

Supplementation of INDO, a COX inhibitor, induced the stimulation of cortisol production by EPA and ARA observed in the former set of experiments, with cortisol absolute values not being different from those of the control (Fig. 3). However, a significantly higher ($P<0·05$) cortisol peak was obtained when the tissue was incubated with DHA, despite the addition of INDO (Fig. 3).

Comparison of cortisol stimulation factors when INDO was added showed a significantly higher ($P<0·05$) cortisol secretion in the tissue supplemented with EPA, ARA and DHA (Fig. 4). Thus, the stimulation factor of cortisol was 7·83±3·31 when tissue was supplemented with EPA, 6·97±4·56 with ARA, 13·67±2·66 with DHA and only 1·58±0·45 for control.

In addition, the comparison of cortisol stimulation factors between experiments with or without INDO showed that the addition of INDO significantly decreased ACTH-stimulated cortisol secretion in all the treatment use of this COX inhibitor (Fig. 5). However, this impaired stimulation of cortisol production was lower in the DHA-supplemented group in which INDO caused a 40·84% reduction in cortisol secretion, giving values that were significantly different ($P<0·05$) compared to EPA treatment where INDO caused

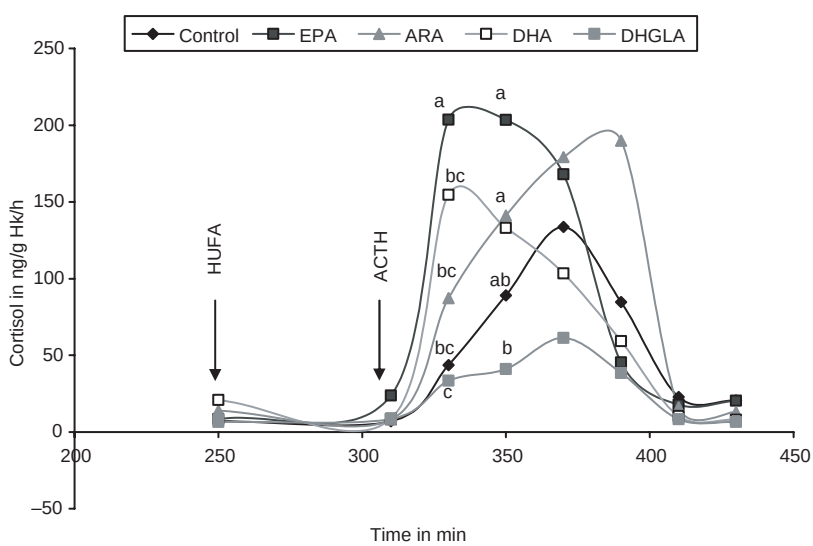

Figure 1 Absolute cortisol secretion (ng/g Hk/h) by sea bream head kidney (Hk) after ACTH stimulation following incubation with HUFA (different letters for a given time indicate significant difference, $P<0·05$).

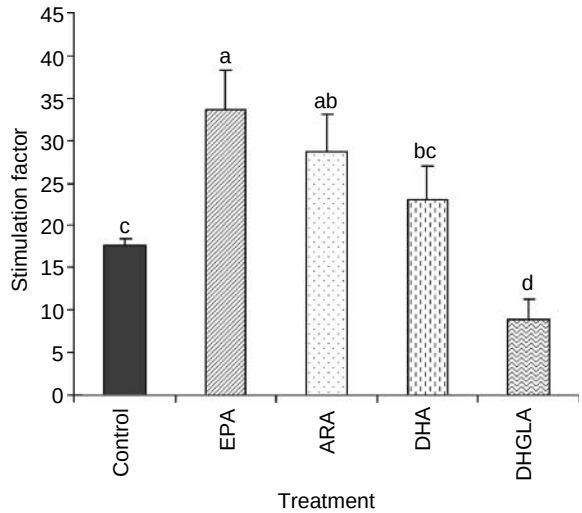

Figure 2 Cortisol stimulation factor in sea bream head kidney after ACTH stimulation following incubation with different HUFA (different letters for different treatments indicate significant difference, $P<0{\cdot}05$ ANOVA).

a 76·76% reduction in cortisol secretion and a 75·71% reduction with ARA treatment.

Discussion

The present study showed the first evidence of the effect of HUFA on cortisol production by ACTH-stimulated interrenal cells in fish. These results are in agreement with the observed modulating effect of dietary fatty acids in sea bream plasma cortisol levels (Montero *et al.* 1998, 2001) and confirm the hypothesis of these authors about the effective action of these fatty acids on the cortisol secretion by the interrenal cells in gilthead sea bream.

Both ARA and EPA promoted ACTH-induced cortisol production in sea bream interrenal cells used in the present experiment. Dietary EPA has been shown to affect fish stress resistance in several species. Although it promoted the growth and survival of the red sea bream (Watanabe *et al.* 1989), gilthead sea bream (Liu *et al.* 2002) and Japanese flounder (Furuita *et al.* 1998), its effects on larval stress resistance seem to depend on species and dietary levels. For instance, elevation of dietary EPA increased red sea bream handling stress resistance (Watanabe *et al.* 1989) and gilthead sea bream resistance to air exposure and temperature shock, but not to salinity stress (Liu *et al.* 2002). On the contrary, too high EPA levels reduced stress resistance to air exposure in Japanese flounder (Furuita *et al.* 1998). ARA has also been shown to affect stress resistance in several fish species. Dietary ARA levels of about 1% dry weight feed are necessary not only for optimum growth and survival of sea bream larvae (Bessonart *et al.* 1999), but also for improved stress resistance after handling (Koven *et al.* 2003, Van Anholt *et al.* 2004). Dietary ARA levels close to those used by these authors did not affect the handling of stress resistance in Japanese flounder, whereas higher ones reduced larval stress resistance (Furuita *et al.* 1998).

These differences of the effects of dietary EPA or ARA on stress resistance in different species may also be related to different ratios among these fatty acids, since both are competing substrates for cyclooxygenase enzymes (Izquierdo *et al.* 2001). For instance, in Atlantic salmon, alteration in the dietary ratio of n-3/n-6 fatty acids has been shown to prevent stress susceptibility to transport (Bell *et al.* 1991). The present

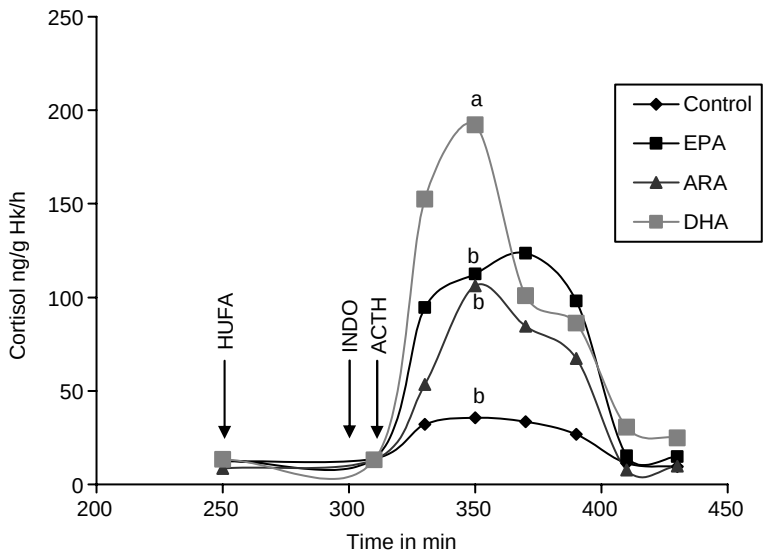

Figure 3 Absolute cortisol secretion (ng/g Hk/h) by sea bream head kidney (Hk) after ACTH stimulation following incubation with different HUFA and INDO supplementation (different letters for a given time indicate significant difference, $P<0{\cdot}05$).

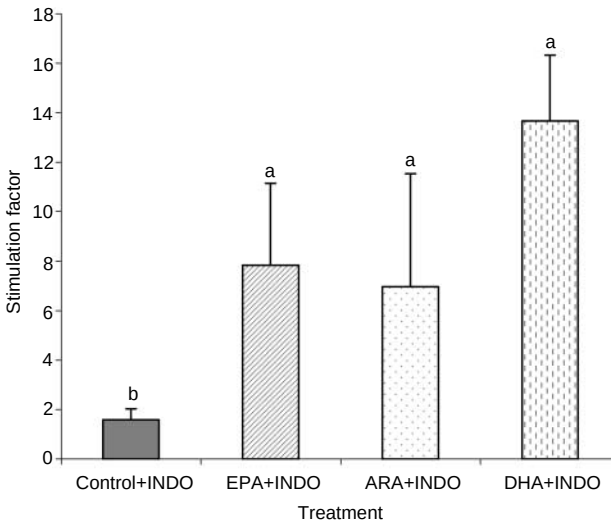

Figure 4 Cortisol stimulation factor in sea bream head kidney after ACTH stimulation following incubation with different HUFA and INDO supplementation (different letters for indicate significant differences among treatment).

study shows the first evidence found in fish that cyclo-oxygenase-derived metabolites are involved in ACTH-induced cortisol release by interrenal cells. The strong reduction of cortisol release caused by INDO addition in EPA and ARA supplemented groups suggested that the effect of these fatty acids was, at least partly, mediated by their cyclooxygenase-derived metabolites. Both fatty acids have been found to be good precursors of cyclooxygenase-derived PG in fish (Bell *et al.* 1994*a*, Ganga *et al.* 2005). In turn, cyclooxygenase-derived PG have been shown to increase *in vitro* cortisol release in interrenal tissue of female frogs during

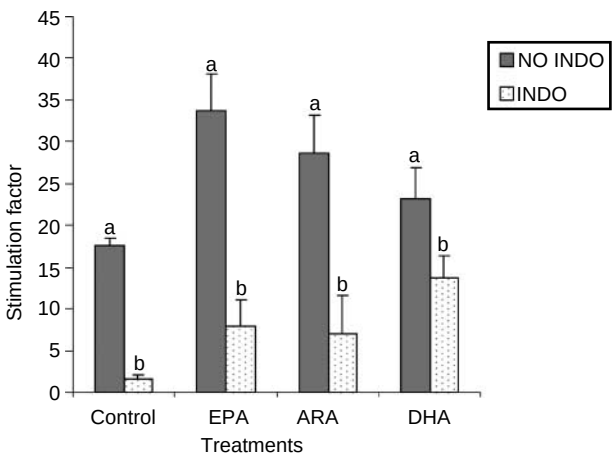

Figure 5 Comparison of cortisol stimulation factor in sea bream head kidney after ACTH stimulation following incubation with different HUFA and including (filled bars) or not (dotted bars) INDO supplementation (different letters for control or each fatty acid incubation indicates significant differences by INDO addition).

ovulation (Gobbetti & Zerani 1993) and in human adrenal cells as well (Vakharia & Hinson 2005).

Interestingly, DHA stimulation of ACTH-induced cortisol production was lower than that caused by EPA or ARA. Besides, the inhibitory effect of INDO in the DHA-supplemented group was not so marked as in the other treatments, suggesting that the action of DHA in cortisol release from ACTH-stimulated interrenal cells is less dependent on COX metabolites in gilthead sea bream. Indeed, this fatty acid is a poorer substrate for COX than EPA or DHA. The action of DHA on interrenal cells, whether it is direct or mediated by its lipoxygenase derivatives, still has to be elucidated since lipoxygenase metabolites have been shown to modify the hormone-induced release of cortisol in mammal adrenal tissues (Wang *et al.* 2000, Yamazaki *et al.* 2001). Using nordihydroguaiaretic acid, a lipoxygenase inhibitor, cortisol secretion was inhibited in response to ACTH in bovine adrenocortical cells (Wang *et al.* 2000).

DHA has long been known for its high value as an essential fatty acid for marine fish (Watanabe 1982), particularly during larval stages (Izquierdo *et al.* 1989) when it invariably promotes growth, survival and stress resistance to a higher extent than EPA or ARA in all the studied species (Watanabe *et al.* 1989, Kanazawa 1997, Rodríguez *et al.* 1997, Furuita *et al.* 1999, Izquierdo *et al.* 2005). In gilthead sea bream, dietary deficiencies of n-3 HUFA and especially DHA have been shown to increase plasma cortisol levels after both acute (net chasing) and chronic (high stocking density) stress (Montero *et al.* 1998, 2001). Besides, imbalances in the dietary n-3/n-6 fatty acids ratio induced by the inclusion of vegetable oils in the diet have been shown to alter the release of cortisol after stress in this species (Montero *et al.* 2003) and in other species such as chinook salmon (Welker & Congleton 2003). The role of dietary oils on stress response in fish remains unclear, but results indicate that dietary fatty acids could be regulating the *in vivo* stress response through the mechanisms discussed above. Moreover, vegetable oils in fish diets have been shown to regulate COX-derived eicosanoids directly (Ganga *et al.* 2005). Dietary supplementation of other fatty acids such as ARA seems to be affecting plasma cortisol levels after stress (Van Anholt *et al.* 2004), although the effect on cortisol release *in vivo* is dose dependent, since high levels of ARA in diet seem to be detrimental to chronic stress resistance in larval gilthead sea bream (Koven *et al.* 2003).

Concentrations of fatty acids used in the present study were those providing the maximum cortisol stimulation factor (50 μM). However, higher concentrations reduced and even inhibited cortisol secretion (Acerete L, Ganga R, Tort L & Izquierdo MS. unpublished results), suggesting a concentration dependency in the type of effect of these fatty acids. This is the case in other organs such as testicles where medium concentrations of ARA (3-30 μM) induced testosterone production in testicular cells of male sea bass, whereas high concentrations (300 μM) inhibited it (Asturiano 1999). Despite the fact that no previous data has been published on *in vitro* exposures of fish interrenal cells to fatty acids, cytotoxic

effects at the membrane level have been found in mammalian tissues, including renal cells (Zager *et al.* 1997). Particularly, excess of ARA and its derived eicosanoids has been found to cause apoptosis associated with oxidative stress in human leukocytes (Pompeia *et al.* 2002). Studies are being conducted at present to elucidate the effect of low and high physiological concentrations of fatty acids on ACTH-induced cortisol release by fish interrenal cells. Although this superfusion method is widely used for studies of interrenal tissue in fish (Rotllant *et al.* 2001), it prevents the possible control of blood flow by COX-derived prostanoids. Nevertheless, new results have shown the vasoregulatory function of COX-derived products in fish (Stensløkken *et al.* 2002), none of them relate though to interrenal tissues.

In summary, the role of ARA and EPA in the ACTH-induced release of cortisol from gilthead sea bream interrenal cells seems to be partly related to COX-derived metabolites dependent on ARA and EPA, whereas the role of DHA seems to be dependent on other factors. Both EPA and ARA as single supplemented fatty acids increased the ACTH-induced cortisol release from gilthead sea bream interrenal cells whereas the effect of DHA was weaker. A well-balanced supplementation of these three fatty acids could be necessary to regulate cortisol release from interrenal cells in gilthead sea bream.

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