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Arachidonic acid reduces the stress response of gilthead seabream *Sparus aurata* L.

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Summary

In this study the influence of the dietary level of the fatty acid arachidonic acid (ArA, 20:4n-6) was determined on the acute stress response and osmoregulation of adult gilthead seabream *Sparus aurata* L. Seabream were fed a diet containing either 0.9% or 2.4% of total fatty acids as ArA for 18 days before being subjected to a 5 min period of net confinement. Prior to this stressor, a subgroup of fish from both dietary treatment groups was treated with acetylsalicylic acid (ASA), an irreversible blocker of cyclo-oxygenase (COX). This would indicate whether any effects were caused by an enhanced synthesis of prostaglandins derived from ArA. The highest ArA levels were found in the kidneys, and these were further enhanced by dietary ArA-supplementation. In gill tissues, there were significant changes in all selected fatty acid classes 24 h after confinement, except for the docosahexaenoic acid (DHA, 22:6n-3) : eicosapentaenoic acid (EPA, 20:5n-3) ratio. ArA feeding strongly reduced the cortisol response to confinement, which was partially counteracted by ASA treatment. ArA also attenuated the stress-associated increase in plasma osmolality and, in combination with ASA, enhanced the osmolality and plasma chloride levels, but reduced plasma sodium levels after confinement. Furthermore, ArA enhanced the branchial Na^+, K^+-ATPase activity both before and after confinement, whereas feeding ASA diminished this effect. It appeared that the effects of ArA-supplementation could not always be ascribed to an increase in prostaglandin synthesis. It is advisable to determine the long-term effects of replacing fish oils in commercial diets with vegetable oils that contain no long-chain fatty acids, particularly in carnivorous/marine species with low fatty acid elongation and desaturation activities. The effects of a low dietary intake of ArA (and other polyunsaturated fatty acids) should be studied over a longer term, taking into account any consequences for the health of the fish.

Key words: acetylsalicylic acid, arachidonic acid, cyclooxygenase, cortisol, eicosanoid, osmoregulation, prostaglandins, gilthead seabream, *Sparus aurata*.

Introduction

Like most marine fish species studied so far, gilthead seabream *Sparus aurata* L. have an essential requirement for the polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ArA, 20:4n-6) to enable optimal growth and survival. However, the desaturase and elongase activities necessary to synthesize these long chain PUFA from shorter carbon chain precursors are very low, so all three essential PUFA have to be provided in the diet (Mourente and Tocher, 1994; Tocher and Ghioni, 1999; Bell and Sargent, 2003). Dietary enrichment with ArA not only improved growth and survival in larval seabream (Bessonart et al., 1999), but also reduced the stress-related mortality that occurred after handling and tank transfer (Koven et al., 2001). However, higher levels of ArA (~6 mg ArA g^{-1} dry mass in *Artemia nauplii*) became detrimental when the larvae were under conditions of chronic stress (Koven et al., 2003).

ArA is selectively incorporated into cellular phospholipids, especially phosphatidylinositol, despite the general abundance of n-3 PUFA in phospholipids of fish tissues (Linares and Henderson, 1991). While the structural contribution of ArA to the membrane appears to be limited, ArA can be rapidly converted into eicosanoids, which can function as endocrine, paracrine and/or autocrine modulators of secretory mechanisms in various organs (Bihoreau et al., 1990; Lands, 1991). The conversion process begins when stress-related triggers induce the release of ArA within 5–60 s by activating phospholipases, mainly PLA_2 (Axelrod et al., 1988; Smith, 1989; Buschbeck et al., 1999). It is often assumed that the effects of dietary ArA on fish are mainly due to its function as a precursor to these eicosanoids, particularly the prostaglandins of the 2-series (Beckman and Mustafa, 1992; Bell et al., 1995; Harel et al., 2001; Bell and Sargent, 2003). The prostaglandins are known to control a wide variety of physiological processes

in mammals as well as in fish, including respiratory and cardiovascular output (McKenzie, 2001), ovulation and spawning behavior, oocyte maturation, nervous system function, osmoregulation (Mustafa and Srivastava, 1989) and immune functions (Rowley et al., 1995). Furthermore, prostaglandins can modulate the sensitivity of the hypothalamus–pituitary–adrenal (HPA) axis in mammals at various levels and alter the release of cortisol and corticosterone in the stress response (Zacharieva et al., 1992; Nye et al., 1997; Wang et al., 2000). In teleost fish the release of cortisol is controlled by the analogous hypothalamic–pituitary–interrenal (HPI) axis (Wendelaar Bonga, 1997). Several studies have found indications that ArA, and other PUFA, are involved in the regulation of cortisol release in fish as well (Gupta et al., 1985; Wales, 1988; Harel et al., 2001; Koven et al., 2003). Moreover, dietary supplementation with ArA clearly reduced the cortisol response of seabream larvae to air exposure (Van Anholt et al., 2004).

The present study was designed to establish the influence of the dietary level of ArA on the response of adult seabream to an acute stressor. This could be relevant as attempts are being made to replace fish oil by vegetable oils as a source of lipids in the production of fish feeds. These oils contain no C20 and C22 PUFA, which might lead to deficiencies, particularly in marine fish that depend on a dietary source of these long-chain fatty acids (Bell, 1998; Bell and Sargent, 2003). More specifically, several studies have indicated that effects of dietary ArA during the stress response could be attributed to an increased production of the 2-series prostaglandins (see, for instance, Gupta et al., 1985; Harel et al., 2001; Van Anholt et al., 2003). Acetylsalicylic acid (ASA) is an irreversible blocker of the COX pathway, which is responsible for the conversion of ArA into prostaglandins (Smith, 1989). The administration of ASA was an effective and stress-free method for the *in vivo* inhibition of the COX pathway in Mozambique tilapia *Oreochromis mossambicus* (Van Anholt et al., 2003). Therefore, subgroups from each of the low- and high-ArA treatments were fed ASA before being subjected to an acute stressor to investigate the role ArA and prostaglandins in the stress response.

Materials and methods

The experiments described in this study were conducted in accordance with the current law on animal experimentation in the Netherlands.

Fish

Male and female gilthead seabream *Sparus aurata* L. were obtained from a commercial hatchery (Vendée Aquaculture, La Faute sur Mer, France) at an approximate mass of 5 g. They were raised in the laboratory of the University of Nijmegen and fed commercial pellets for seabream and seabass (crude protein 46%, total lipid 20%, ash 10%, water 5%; Filia LDX, Trouvit, Fontaine Les Vervins, France) at 2% of their body mass. The fatty acid profile of these pellets is listed in Table 1. Fish were kept in recirculated synthetic saltwater (Aqua Medic Sea Salt, Bissendorf, Germany) of 34‰ and 20°C with a 12 h photoperiod. The recirculation system was equipped with a protein skimmer, biological filter and UV-sterilizing unit.

Experimental diets

Two types of 4 mm pelleted diets (The National Center for Mariculture, Eilat, Israel) were used. These diets were identical in their protein, lipid and micronutrient levels (crude protein 45%, total lipid 19%, ash 11% and water 8%), but differed in

Table 1. *Fatty acid profiles of the standard diet and the two experimental diets, the diet with a low level of arachidonic acid (low-ArA) and pellets enriched with ArA (high-ArA)**

Diet	Standard	Low-ArA	High-ArA
Saturates	29.4±0.1	30.3±0.3	30.2±0.3
Mono-unsaturates	36.1±0	33.1±0.1	32.7±0.3
Polyunsaturates	34.8±0.1	36.9±0.2	37.4±0.5
n-3 Polyunsaturates	27.9±0.1	22.5±0.3	20.7±0.6
n-6 Polyunsaturates	4.0±0.1	12.3±0.1	14.8±0
n-3/n-6	7.1±0.1	1.8±0	1.4±0
α-Linolenic acid (α-LNA,18:3n-3)	1.3±0.1	1.2±0	1.2±0
Linoleic acid (LA, 18:2n-6)	2.9±0	10.9±0.1	11.6±0
α-LNA/LA	0.4±0	0.1±0	0.1±0
Docosahexaenoic acid (DHA, 22:6n-3)	11.9±0.1	11.1±0.2	10.5±0
Eicosapentaenoic acid (EPA, 20:5n-3)	9.9±0	7.0±0.1	6.7±0
Arachidonic acid (ArA,20:4n-6)	0.6±0	0.9±0	2.4±0
DHA/EPA	1.2±0	1.6±0	1.6±0
DHA/ArA	20.7±1.1	12.5±0.3	4.3±0.1
EPA/ArA	17.3±0.6	7.8±0.1	2.7±0.1

*See Materials and methods.

Fatty acids were determined in duplicate subsamples and are expressed as percentages of total fatty acids. Values are means ± S.E.M.

their fatty acid composition. In the 'low-ArA' pellets, fish oil (predominantly capelin oil) was incorporated at 10% of the pellet dry mass, which contained moderate levels of DHA, EPA and linoleic acid (LA), while having low levels of ArA. In the other diet (high-ArA diet) 10% of the fish oil fraction was replaced with ARASCO (Martek Biosciences, Columbia, MD, USA) to give a final ArA level of 2.4±0.0% of total fatty acids, compared to 0.9±0.0% of total fatty acids in the low-ArA diet. This corresponded to 1.9 mg ArA g^{-1} dry mass and 0.6 mg ArA g^{-1} dry mass in the high- and low-ArA pellets, respectively. ARASCO is a highly purified ArA-rich oil extracted from the fungus *Mortierella alpina*, containing ~40% ArA of total fatty acids. The fatty acid compositions of the pellets were determined according to Koven et al. (2001) and the results are listed in Table 1.

Experimental design and stress challenge

In two consecutive trials, 160 seabream (80.7±1.2 g) from the same cohort were used. Each trial included 80 seabream, which were equally divided according to mass into eight groups of ten fish. Four groups of ten fish were fed low-ArA containing pellets, while the other four groups were fed the high-ArA containing pellets. All fish were fed daily rations of 2% of their body mass for a period of 18 days. At the end of this period, two groups of ten fish from each treatment group received three doses of ASA in 2 days (100 mg kg^{-1} body mass, based on mean mass of the group). ASA was incorporated into gelatin capsules together with crushed pellets, which were eaten voluntarily within 5 min, according to the method described by Van Anholt et al. (2003). On the second day, approximately 4 h after receiving their last meal or dose of ASA, several fish were sampled for baseline values. Our previous observations in tilapia indicated that plasma salicylate levels peaked around 5 h after administration before declining to basal levels over a 15 h period (Van Anholt et al., 2003). Confinement was therefore timed in such a way that the fish would experience any stressor-induced peak in plasma cortisol whilst plasma salicylate levels were still increasing. As plasma salicylate measurements are corrected for blank readings (<0.14 mmol l^{-1}), the salicylate in control fish was probably of dietary origin such as vegetable material, a known source of natural salicylates. The remaining fish were immediately subjected to the stress challenge, which consisted of 5 min of confinement in a submerged dip-net. After confinement they were released back into the aquarium and sampled after 20 min, 60 min or 24 h. Fish were anaesthetized in a 2% 2-phenoxyethanol solution and blood samples were immediately collected from the caudal vein using heparinized needles. Plasma samples were collected after centrifugation (16 000 ***g***, 5 min) and stored at –20°C until analysis. Tissue samples of gills, muscle and kidney were removed immediately and stored at –30°C until analysis. Gill arches were dissected and stored frozen in SEI buffer (300 mmol l^{-1} sucrose, 20 mmol l^{-1} Na_2EDTA, 100 mmol l^{-1} imidazole, adjusted to pH 7.4 with Hepes-Tris) until analysis of Na^+, K^+-ATPase activity.

Plasma measurements

Cortisol levels were determined using a competitive radioimmunoassay (Campro Scientific, Veenendaal, The Netherlands) according to the procedure described in Van Anholt et al. (2003). Plasma glucose levels were determined in duplicates with Sigma's INFINITY glucose reagent according to the manufacturer's protocol (Sigma-Aldrich, Poole, Dorset, UK). Plasma concentrations of lactate were assayed in duplicate by a standard colorimetric assay (735-10; Sigma-Aldrich). Plasma concentrations of sodium, potassium and chloride were determined by flame photometry (Radiometer FLM3, Copenhagen, Denmark) and plasma osmolality was determined in 50 µl undiluted plasma samples using an automatic cryoscopic osmometer (Osmomat 030, Gonotec, Berlin, Germany).

Na^+, K^+-ATPase activity

Branchial filaments were collected and homogenized in 250 µl SEI buffer containing aprotinin (5 µl ml^{-1}), and after centrifugation (10 min at 500 ***g***) supernatants were used for analysis. ATPase activity was determined as the specific release of inorganic phosphate P_i from ATP, using either 12.5 mmol l^{-1} KCl or 1 mmol l^{-1} ouabain in the medium, according to the method described in Van Anholt et al. (2003). The difference between the total ATPase activity and the ouabain-insensitive ATPase activity was designated as the ouabain-sensitive, K^+-dependent Na^+, K^+-ATPase activity and expressed in µmol P_i h^{-1} mg^{-1} protein.

The in vitro *effect of free ArA on Na^+, K^+-ATPase activity*

Gill and kidney homogenates of eight control fish were pooled and the COX inhibitor indomethacin (10 µg ml^{-1} homogenate) was added to prevent prostaglandin synthesis. The Na^+, K^+-ATPase activity was assayed using free ArA in both incubation media, containing KCl or ouabain, respectively. Stock solutions of free ArA (Sigma, St Louis, MI, USA) were prepared in 100% methanol and 100× diluted with the reaction media to their final concentrations, 1–100 µmol l^{-1} at 1% (v/v) methanol. Enzyme activity in the presence of added ArA was expressed as a percentage of the value in the 1% methanol controls.

Statistics

Initial analysis revealed no differences between the two trials and the results were therefore combined and treated as one experiment. All data are expressed as means ± S.E.M. Levene's Tests for homogeneity of variances indicated that log-transformation was required for cortisol data and arcsine transformations were performed on the percentage data of fatty acids to achieve homogeneity of variance. Three-way analyses of variance (ANOVAs) were performed to determine the effects of the factors ArA feeding, ASA-administration and time of sampling. *Post hoc* multiple comparison tests (Tukey's HSD) were used to determine which time points differed significantly (SPSS software, version 11.5). Effects of free ArA in the *in vitro* Na^+, K^+-ATPase test were compared

with repeated-measures ANOVA against the activity at 1% methanol. 5% level of probability was accepted as statistically significant.

Results

No mortality was observed during the experiments, nor was there any difference in growth between treatments at the end of the feeding trials. During the 18 days of the feeding trial the fish increased in mass from 80.7±1.2 g to 89.1±1.5 g (means ± S.E.M.). The gelatin capsules containing ASA were eaten voluntarily within 5–10 min and did not reduce appetite when fed simultaneously with the food pellets. Macroscopic evaluation revealed no hemorrhages at the end of the experiments.

ArA feeding had no effect on plasma salicylate levels. Salicylate levels were significantly elevated in seabream that received ASA (P<0.001; Table 2). In all four treatments salicylate levels had dropped below those of all other time points after 24 h (P<0.001). Furthermore, a significant interaction existed between ASA administration and time of sampling (P=0.013).

Fatty acid profiles of gill, kidney and muscle tissues prior to net confinement (t=0) and 24 h after the stressor (t=24 h) are listed in Table 3, and significant effects are presented in Table 4. Feeding the ArA-supplemented diet for 18 days to seabream significantly increased (P=0.028) the levels of ArA in kidneys and reduced the level of linoleic acid (P=0.046), as well as increased the saturated fatty acid levels (P=0.049). ArA feeding decreased the DHA/ArA and EPA/ArA ratios in kidneys (P<0.001), while reducing the DHA/ArA ratio in gill tissues (P=0.035). In the gills, all major fatty acids were significantly different 24 h after confinement, exhibiting either increasing or declining percentages, except for the DHA/EPA ratio (for details, see Table 4). At the same time the levels of saturated fatty acids in the kidneys were significantly (P=0.029) enhanced by confinement, where muscle tissues exhibited a significant increase (P=0.042) in the DHA/EPA ratio after 24 h. There were no significant interactions between factors in any of tissues tested.

Table 2. *Plasma levels of salicylate in plasma of gilthead seabream, after they were fed a diet low in arachidonic acid (low-ArA) or a diet enriched with ArA (high-ArA)*

Treatment*	Time† (min)	Salicylate (μmol l^{-1})
Low-ArA	0	116.5±9.9
	20	118.9±15.0
	60	103.5±20.5
	24 h	13.8±12.0
Low-ArA+ASA	0	290.4±46.3
	20	335.3±60.0
	60	200.6±39.7
	24 h	42.3±5.8
High-ArA	0	95.0±12.2
	20	121.3±22.4
	60	127.4±54.1
	24 h	46.8±5.7
High-ArA+ASA	0	282.4±71.5
	20	277.2±62.9
	60	402.2±136.8
	24 h	38.2±13.9

*Dietary ArA had no effect on salicylate levels, which were significantly higher in seabream that received ASA (P<0.001).

†In all four treatments, plasma salicylate levels were significantly lower after 24 h than at all other time points (P<0.001). The interaction between ASA administration and time of sampling was significant (P=0.013).

Fish were exposed to a stressor (5 min confinement in a dip-net) and samples were collected just before (t=0) and at intervals after confinement (20 min, 60 min, 24 h). The groups that were treated with acetylsalicylic acid (+ ASA) were confined 4 h after the last dose of 100 mg ASA kg^{-1} body mass.

Values are means ± S.E.M. of 10 fish.

Within 20 min after confinement plasma cortisol levels increased substantially in all treatments, resulting in significantly higher plasma levels compared to all other time-points (P<0.001; Fig. 1). After 60 min, cortisol levels were no longer significantly elevated compared to the pre-confinement levels. The ArA-supplemented seabream exhibited a significantly lower cortisol response (P=0.012) compared to the fish fed the 'low-ArA' diet. In addition, ASA significantly affected the cortisol response (P=0.045). In the low-ArA fed seabream cortisol levels were reduced by ASA, whereas in the high-ArA fed fish cortisol levels were enhanced by ASA. There were no significant interactions between ArA, ASA, and/or the time of sampling.

The fish responded to confinement with a significant increase (P<0.001) in plasma glucose at t=20 min, but glucose was no longer significantly different from pre-confinement levels after 60 min (Fig. 2). Glucose levels were significantly influenced by the administration of ASA (P=0.015). Glucose levels were markedly reduced after ASA administration in the seabream fed the low-ArA diet, while glucose levels were not influenced by ArA-supplementation, nor were there any significant interactions between ArA, ASA, and/or time of sampling.

Plasma lactate levels increased in all treatments 20 min after confinement (P<0.001; Fig. 3). 1 h later, lactate levels were still significantly higher than prior to confinement (P=0.029), as well as at 20 min and 24 h afterwards (P=0.001 and 0.032, respectively). The dietary level of ArA had no effect on plasma lactate levels, whereas the administration of ASA significantly (P=0.024) altered the lactate response, mainly in the high-ArA fed seabream. This was also indicated by the significant interaction between all three factors, ArA, ASA and time of sampling (P=0.032).

Plasma osmolality increased considerably within 20 min in all treatments (P<0.001), until at least 60 min (P=0.008), but after 24 h these levels were no longer significantly different from levels prior to confinement (Fig. 4). Plasma osmolality was not affected by ArA-supplementation, but the effect of ASA was significant (P=0.020). In the low-ArA group plasma

Table 3. *Overview of (categories of) fatty acids expressed as percentages of total fatty acids of gill, kidney and muscle tissues*

	Low-ArA		Low-ArA+ASA		High-ArA		High-ArA+ASA	
	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
(A) Gill								
Saturates	36.1±2.7	39.9±0.2	34.4±2.9	39.0±2.3	34.4±3.1	42.6±1.7	35.6±2.7	41.2±0.8
Mono-unsaturates	44.7±1.9	50.0±3.1	42.7±1.9	47.8±1.8	43.2±1.6	46.5±1.4	44.2±1.7	47.6±0.9
Polyunsaturates	19.4±4.6	9.9±0.8	23.0±4.4	13.3±3.9	22.6±4.7	10.9±1.7	20.4±4.4	11.3±1.7
n-3 Polyunsaturates	13.7±3.9	5.3±0.5	16.7±3.7	8.3±3.3	16.2±3.9	6.6±1.4	14.7±3.8	6.5±1.2
n-6 Polyunsaturates	3.7±0.5	3.1±0.2	4.6±0.5	3.6±0.6	4.6±0.6	2.9±0.2	4.1±0.6	3.3±0.5
n-3/n-6	3.4±0.6	1.7±0.1	3.6±0.6	2.1±0.4	3.3±0.4	2.2±0.4	3.4±0.4	1.9±0.1
α-LNA	0.6±0.1	0.4±0	0.8±0.2	0.4±0.1	0.7±0.1	0.3±0.1	0.6±0.1	0.4±0
LA	2.9±0.4	2.4±0.2	3.4±0.4	2.5±0.4	3.3±0.5	2.0±0.1	1.0±0.4	2.3±0.3
α-LNA/LA	0.2±0	0.2±0	0.2±0	0.2±0	0.2±0	0.2±0	0.2±0	0.2±0
DHA	7.1±2.2	2.8±0.4	7.9±2.1	4.2±1.7	8.1±2.0	2.8±0.8	7.8±2.0	3.3±0.7
EPA	3.4±1.0	1.3±0.1	4.6±1.1	2.1±0.9	4.1±1.0	2.4±0.9	3.7±0.9	1.5±0.3
ArA	0.5±0.1	0.4±0.1	0.6±0.1	0.5±0.1	0.8±0.1	0.5±0	0.7±0.1	0.6±0.1
DHA/EPA	2.1±0.1	2.2±0.1	1.8±0.3	2.0±0.1	2.0±0	1.8±0.4	2.1±0.1	2.2±0.1
DHA/ArA	13.5±2.1	7.4±0.3	13.3±2.4	7.8±1.7	10.0±1.1	6.1±1.5	11.3±0.7	6.0±0.3
EPA/ArA	6.4±0.9	3.4±0.2	8.9±3.0	4.0±0.9	5.0±0.6	4.8±1.6	5.4±0.4	2.8±0.1
(B) Kidney								
Saturates	40.4±7.0	33.8±8.2	36.9±3.3	51.2±7.8	39.4±3.8	44.6±4.1	44.9±3.8	53.9±5.0
Mono-unsaturates	26.4±1.1	30.3±3.0	28.2±2.3	23.4±1.5	25.5±3.4	26.8±4.8	26.5±1.5	23.1±4.9
Polyunsaturates	33.4±5.8	36.0±8.8	35.0±2.2	25.8±6.5	35.2±2.8	29.1±5.1	28.8±3.5	23.1±1.2
n-3 Polyunsaturates	18.1±1.5	23.7±6.1	21.1±1.4	14.8±5.2	21.4±1.5	14.8±3.9	17.0±2.5	12.7±1.5
n-6 Polyunsaturates	13.2±3.8	10.2±2.3	11.4±0.8	8.8±1.5	12.0±1.2	11.8±1.2	9.9±1.1	8.7±0.6
n-3/n-6	1.5±0.3	2.3±0.1	1.9±0.1	1.6±0.4	1.8±0.1	1.2±0.3	1.7±0.2	1.5±0.2
α-LNA	0.6±0.1	0.8±0.2	0.6±0	0.6±0.2	0.7±0.3	0.4±0.1	0.7±0.3	0.5±0.1
LA	6.1±1.4	5.2±1.1	5.5±0.5	4.3±0.7	5.1±0.3	4.4±0.4	4.3±0.6	3.9±0.4
α-LNA/LA	0.1±0	0.2±0.1	0.1±0	0.1±0	0.1±0	0.1±0	0.1±0	0.1±0
DHA	11.5±1.1	15.3±4.1	13.3±1.0	9.4±3.5	13.9±1.0	9.8±2.7	11.1±1.6	7.1±1.3
EPA	4.0±0.2	5.4±1.6	4.9±0.3	3.3±1.1	4.6±0.4	3.0±0.8	3.8±0.4	3.1±0.1
ArA	3.1±1.1	2.8±0.8	2.6±0.3	2.0±0.5	4.6±0.4	3.6±0.5	3.2±0.7	3.1±0.4
DHA/EPA	2.9±0.1	2.9±0.1	2.7±0.1	2.8±0.3	3.1±0.1	3.3±0.1	2.9±0.2	2.3±0.4
DHA/ArA	4.4±1.1	5.8±0.4	5.2±0.3	4.2±0.9	3.1±0.2	2.6±0.6	3.6±0.4	2.4±0.5
EPA/ArA	1.5±0.5	2.0±0.1	1.9±0.1	1.5±0.3	1.0±0.1	0.8±0.2	1.3±0.1	1.1±0.2
(C) Muscle								
Saturates	28.2±0.4	31.8±3.1	29.3±0.5	29.9±0.8	28.4±0.7	28.1±0.1	27.8±0.1	27.7±0.2
Mono-unsaturates	25.8±2.0	22.0±1.0	24.1±1.2	24.9±1.0	24.8±1.5	23.2±0.7	26.0±2.3	22.7±1.3
Polyunsaturates	46.3±1.7	46.5±3.0	46.9±1.3	45.5±1.2	47.1±1.0	49.0±0.8	46.5±2.2	49.7±1.2
n-3 Polyunsaturates	38.4±1.9	39.2±2.9	39.0±1.1	37.5±1.3	39.0±0.9	41.4±0.7	39.1±2.1	42.0±1.2
n-6 Polyunsaturates	6.2±0.3	6.0±0.4	6.6±0.3	6.5±0.4	6.5±0.1	6.0±0.1	5.8±0.2	6.4±0.6
n-3/n-6	6.2±0.5	6.6±0.4	6.0±0.2	5.8±0.4	6.0±0.2	6.9±0.1	6.8±0.3	6.8±0.7
α-LNA	0.6±0.1	0.6±0.1	0.6±0	0.7±0.1	0.6±0.1	0.4±0.1	0.6±0.1	0.5±0
LA	3.7±0.2	3.4±0.2	3.9±0.1	3.8±0.2	3.5±0.1	3.1±0.1	3.1±0.1	3.3±0.2
α-LNA/LA	0.2±0	0.2±0	0.2±0	0.2±0	0.2±0	0.1±0	0.2±0	0.1±0
DHA	25.2±2.1	26.7±2.5	25.7±1.0	24.5±1.3	25.6±1.1	28.2±0.5	25.7±2.2	28.9±1.4
EPA	8.3±0.2	8.1±0.4	9.0±0.3	8.2±0.3	8.6±0.2	8.8±0.2	8.6±0.2	8.7±0.1
ArA	1.5±0.2	1.6±0.2	1.8±0.2	1.8±0.3	2.0±0	1.9±0.1	1.7±0.2	2.1±0.2
DHA/EPA	3.0±0.2	3.3±0.2	2.9±0.1	3.0±0.2	3.0±0.2	3.2±0.1	3.0±0.2	3.3±0.2
DHA/ArA	17.0±1.4	17.4±0.9	15.0±1.5	15.0±1.8	12.9±0.3	15.1±0.5	15.6±0.9	14.0±1.6
EPA/ArA	5.8±0.6	5.4±0.4	5.4±0.7	5.0±0.5	4.3±0.2	4.7±0.2	5.4±0.6	4.3±0.5

Samples were taken at the end of the feeding period with either the 'low-ArA' or the 'high-ArA' diet, followed by treatment with acetylsalicylic acid when indicated (+ ASA). Samples were collected just prior to (t=0 h) and 24 h after net confinement.

Values represent means ± S.E.M. of 5 fish. Significant differences are shown in Table 4.

Table 4. *Three-way ANOVAs were performed on the arcsine transformed percentage data of the (categories of) fatty acids of the three tissues presented in Table 3*

	Gills			Kidneys			Muscles		
	ArA	ASA	Confinement	ArA	ASA	Confinement	ArA	ASA	Confinement
Saturates	–	–	0.002	0.049	–	0.029			
Mono-unsaturates	–	–	0.002						
Polyunsaturates	–	–	<0.001						
n-3 Polyunsaturates	–	–	<0.001						
n-6 Polyunsaturates	–	–	0.005						
n-3/n-6	–	–	<0.001						
α-LNA	–	–	<0.001						
LA	–	–	0.001	0.046	–	–	0.001		
α-LNA/LA	–	–	0.004						
DHA	–	–	0						
EPA	–	–	0.001						
ArA	–	–	0.027	0.028					
DHA/EPA	–	–	–		–	–	–	–	0.042
DHA/ArA	0.035	–	<0.001	<0.001					
EPA/ArA	–	–	0.008	<0.001					

Significant *P* values ($P<0.05$) of the three tested factors, dietary arachidonic acid (low-ArA *vs* high-ArA), acetylsalicylic acid treatment (no ASA *vs* +ASA), and confinement ($t=0$ *vs* $t=24$ h), are listed.

osmolality was reduced, while in the high-ArA group plasma osmolality was elevated by ASA. Furthermore, there was a strong interaction between ArA and time of sampling ($P<0.001$), as well as between ArA and ASA ($P=0.023$). The interaction of ASA administration with time was also significant ($P=0.001$).

Plasma sodium levels were markedly altered by confinement ($P<0.001$), with a clear increase after 20 min ($P=0.012$), followed by a decrease below pre-confinement levels after 24 h in all treatments ($P<0.001$; Fig. 5A). Neither ArA nor ASA had a significant effect on the sodium response when analyzed as separate factors, yet the interaction between ArA and ASA was significant ($P=0.043$).

In all treatments plasma chloride levels increased within 20 min after confinement ($P<0.001$) and remained significantly elevated for at least 24 h compared to the levels

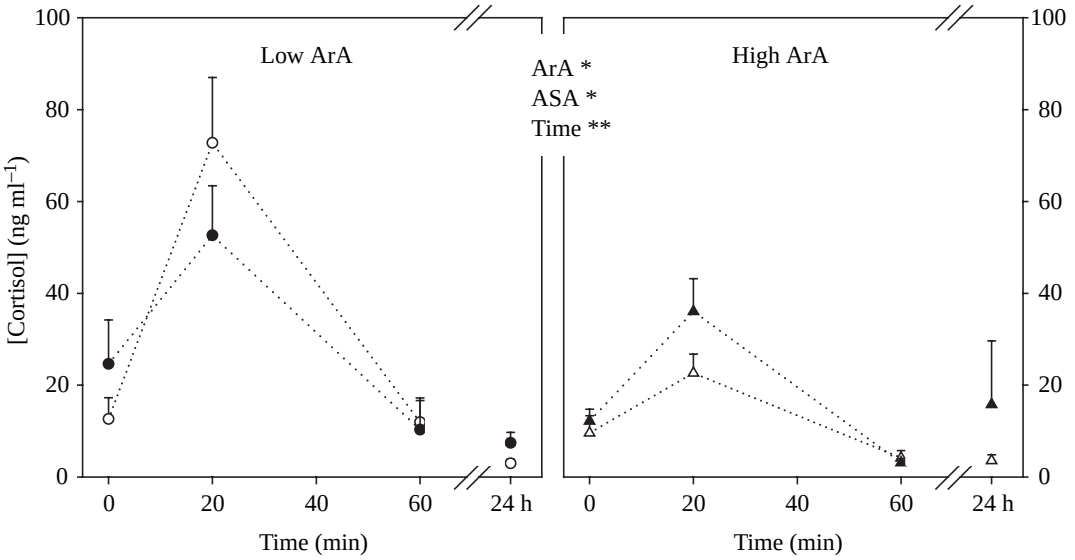

Fig. 1. The effect of a diet low in arachidonic acid (low-ArA) and an ArA-enriched diet (high-ArA) in combination with acetylsalicylic acid (ASA) treatment on plasma cortisol responses to 5 min net confinement. Open circles, low-ArA; filled circles, low-ArA + ASA; open triangles, high-ArA; filled triangles, high-ArA + ASA. Samples were taken from non-stressed fish ($t=0$) and at several intervals after confinement ($t=20$ min, 60 min, 24 h). ASA-treated fish were subjected to confinement 4 h after the last dose of ASA. Each time point represents the mean value ± S.E.M. of 10 fish. The results of both graphs were combined for analysis and significant effects of the factors: ArA supplementation, ASA treatment, time after confinement, and/or interactions are indicated: *$P<0.05$, **$P<0.01$, ***$P<0.001$ (3-way ANOVA).

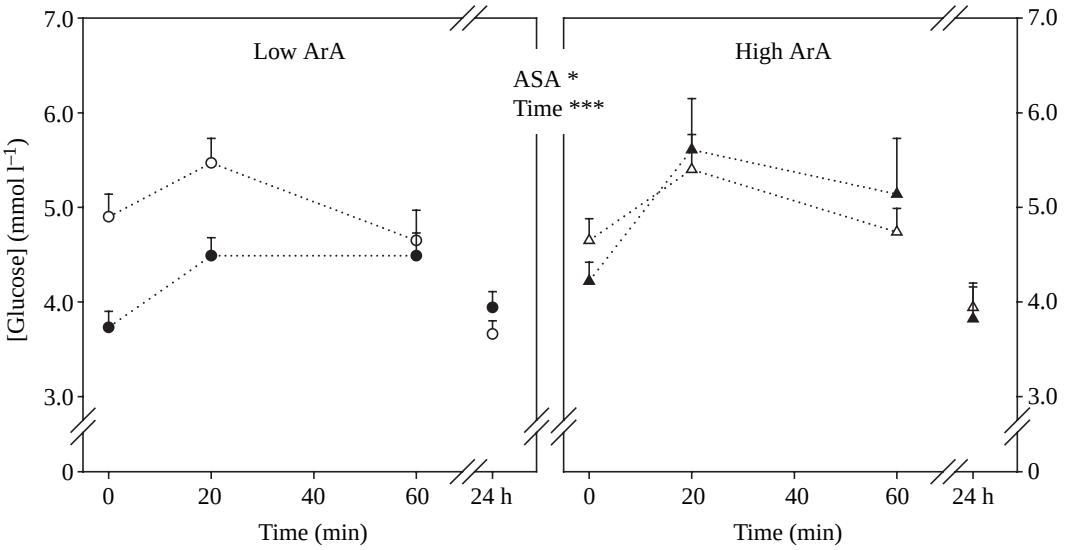

Fig. 2. The effect of ArA, ASA and net confinement on plasma glucose levels. Samples were taken from non-stressed fish (t=0) and at intervals after confinement (t=20 min, 60 min, 24 h). Open circles, low-ArA; filled circles, low-ArA + ASA; open triangles, high-ArA; filled triangles, high-ArA + ASA. ASA-treated fish were subjected to confinement 4 h after the last dose of ASA. Values are means ± S.E.M., N=10.

prior to confinement (P=0.007; Fig. 5B). ArA-supplemented seabream exhibited significantly higher (P<0.001) plasma chloride levels compared to the fish fed the low-ArA diet. In addition, plasma osmolality was significantly elevated (P=0.017) in both dietary treatments after ASA treatment. No significant interactions were found between any of the factors.

Confinement had a significant effect on plasma potassium (P<0.001) with a significant decrease after 20 min (P=0.010) in all treatments. Potassium levels remained lower than pre-confinement levels for at least 24 h (P<0.001; Fig. 5C). ArA-supplementation had no effect on plasma potassium levels, whilst ASA administration resulted in significantly elevated levels of potassium (P=0.041), mainly in the ArA-supplemented seabream.

The branchial Na^+, K^+-ATPase activity was significantly higher (P<0.001) in the ArA-supplemented fish than in the low-ArA group. The ATPase activity was also markedly higher 24 h after confinement than prior to confinement (P=0.039; Fig. 6). In addition, the interaction between ArA and time of sampling was significant (P=0.041), while ASA had no significant effect on the ATPase activity.

Free ArA in the incubation medium reduced the Na^+, K^+-ATPase activity of gill homogenates in a dose-dependent way (P=0.003), except at 10 and 30 μmol l^{-1} ArA (Fig. 7).

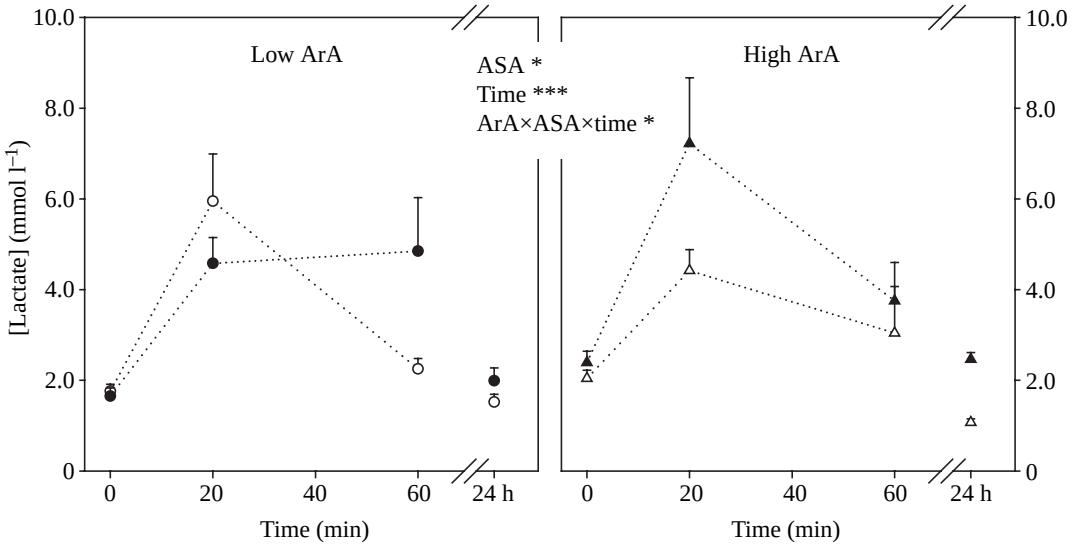

Fig. 3. The effect of ArA, ASA and net confinement on plasma lactate levels. Samples were taken from non-stressed fish (t=0) and at intervals after confinement (t=20 min, 60 min, and 24 h). Open circles, low-ArA; filled circles, low-ArA + ASA; open triangles, high-ArA; filled triangles, high-ArA + ASA. ASA-treated fish were subjected to confinement 4 h after the last dose of ASA. Values are means ± S.E.M., N=10.

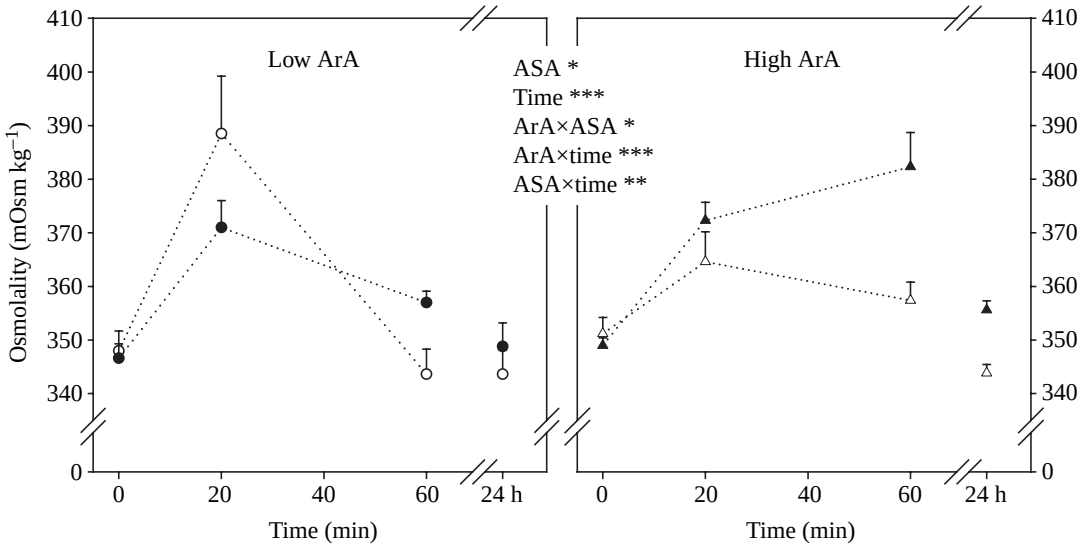

Fig. 4. Changes in plasma osmolality after feeding different diets and ASA administration. Samples were taken just prior to (t=0) and at intervals after net confinement (t=20 min, 60 min and 24 h). Open circles, low-ArA; filled circles, low-ArA + ASA; open triangles, high-ArA; filled triangles, high-ArA + ASA. ASA treated fish were subjected to confinement 4 h after the last dose of ASA. Values are means ± S.E.M., N=10.

Discussion

The incorporation of ArA into gills, intestine, muscle and liver of gilthead seabream has been shown to be most efficient below a dietary ArA level of 2% of total fatty acids, while it dropped considerably above this level (Fountoulaki et al., 2003). Hence, the ArA level of 2.4% of total fatty acids that was used in our 'high-ArA' diet would have been sufficient to enable satisfactory incorporation into the tissues. At the end of the 18-day feeding period the kidneys of seabream fed the ArA-supplemented diet had a significantly higher level of ArA, which also reduced the DHA/ArA and EPA/ArA ratios in those tissues. This relatively short feeding period did not alter the ArA content of muscle and gill tissues. In these tissues the DHA/ArA and EPA/ArA ratios were generally considerably higher than the dietary ratios, signifying that DHA and EPA were preferentially incorporated or retained over ArA. ArA is deposited mainly into the polar lipids, especially at lower dietary levels. Hence, the increase in ArA concentration in kidney tissues apparently reflects changes in the polar lipid fraction, which are more rapid than in the neutral lipid pool (Linares and Henderson, 1991; Fountoulaki et al., 2003). Changes in the fatty acid composition of fish muscle, on the other hand, are thought to result from a dilution effect on neutral lipid pools. This means that changes will result from fatty acids being added to the existing stores as the fish grows and more fatty acids are deposited (Jobling, 2003, 2004; Robin et al., 2003). This process requires a substantial increase in body mass, for which the feeding period in this study seems to have been too short.

On subjecting the seabream to confinement to induce a stress response, the fatty acid composition of the gills was affected, but levels in muscle or kidney tissues hardly at all. After confinement the levels of polyunsaturated fatty acids had decreased in gill tissues, while the monounsaturated as well as the saturated fatty acids exhibited an increase. These changes were not only relative (in percentages of total fatty acids), but were also accompanied by decreasing amounts of EPA, DHA and ArA, when expressed in mg g^{-1} dry mass (data not shown). When synthesizing new membranes, incorporation of readily synthesized saturated and monounsaturated fatty acids is faster than that of PUFA originating from the diet. Hence it is possible that the changes in the gills reflect a higher rate of turnover than in kidneys and muscle. The observed changes might also arise from β-oxidation of the long-chain fatty acids. However, this does not correspond to the general preference in fish tissues for oxidation of monounsaturated fatty acids over saturated fatty acids, which in turn are preferred over polyunsaturated fatty acids (McKenzie et al., 2000).

Feeding the ArA-supplemented diet to gilthead seabream for 18 days was sufficient to substantially diminish the cortisol response after net confinement, compared to the fish fed a diet containing a low level of this fatty acid. This blunted response to stress was very similar to what was found in seabream larvae exposed to another type of acute stress. Both 28- and 50 days post-hatch larvae showed considerable lower peak cortisol levels when they were fed ArA-enriched *Artemia nauplii* prior to a brief exposure to air (Van Anholt et al., 2004). These observations in seabream are in complete contrast to the augmented cortisol response observed in tilapia *Oreochromis mossambicus*, when fed the same diet enriched with ArA (R.D.V.A., F.A.T.S., W.M.K. and S.E.W.B., submitted). Tilapia, like most freshwater species, can convert linoleic acid into ArA, while gilthead seabream has a low ability to form ArA from its precursor. Gilthead seabream apparently became more sensitive to an acute stressor when the dietary intake of ArA was low, emphasizing the importance of ArA in this respect.

To determine whether the observed effects of ArA could be contributed to an enhanced production of prostaglandins, the COX-inhibiting ability of ASA was utilized. A previous study on tilapia verified that the effect of ASA in fish was similar to that in humans, as ASA inhibited the COX activity of kidney homogenates and reduced plasma levels of prostaglandin E_2 (PGE_2; Van Anholt et al., 2003). Feeding the ASA-containing capsules resulted in an almost threefold increase of the plasma salicylate levels in our seabream and the low basal levels of cortisol at the start of the tests confirmed that this was a stress-

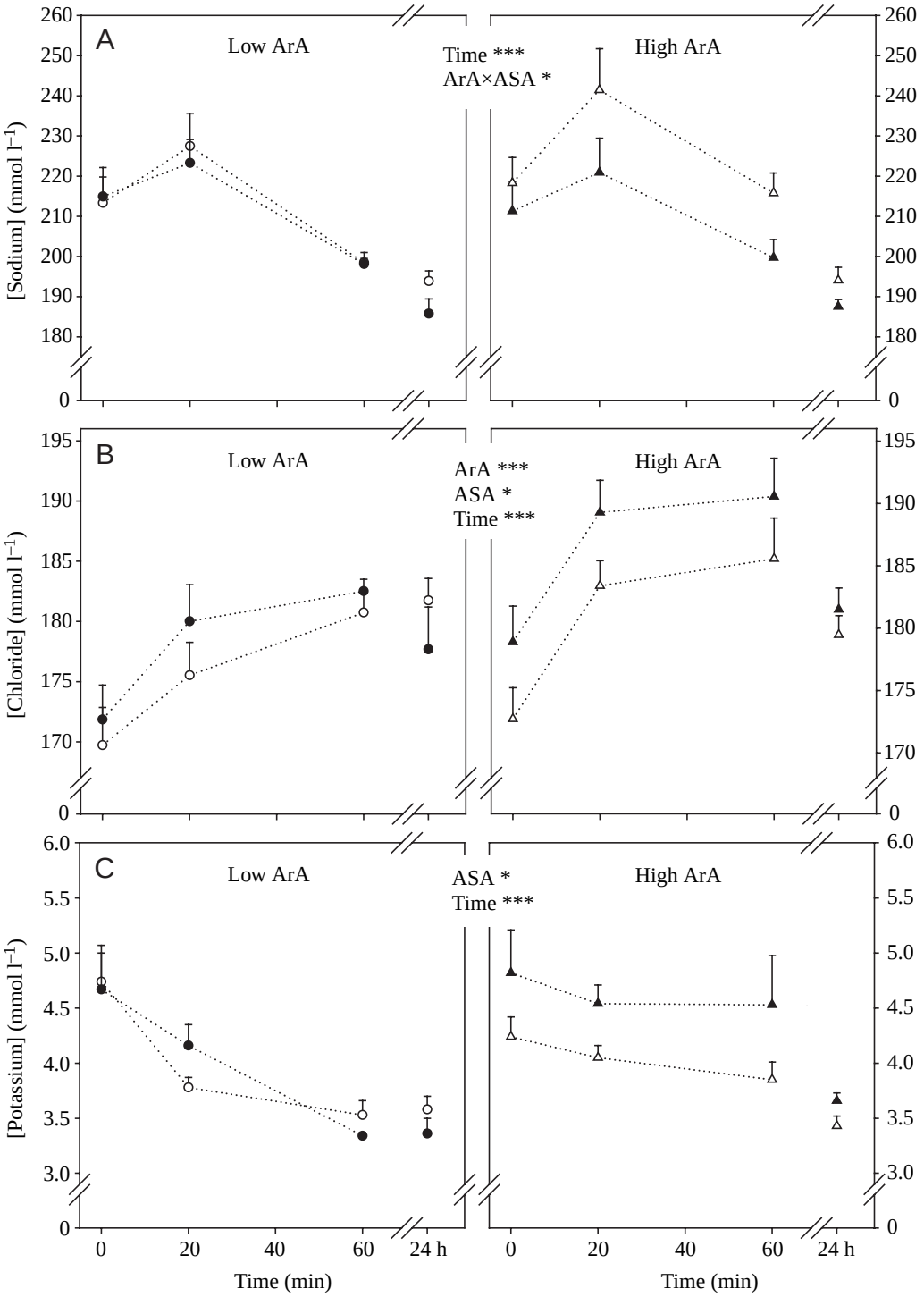

Fig. 5. Changes in plasma ions levels after feeding different diets and ASA administration. (A) Sodium, (B) chloride, (C) potassium. Samples were taken from non-stressed fish (t=0) and after net confinement (for 20 min, 60 min and 24 h): Open circles, low-ArA; filled circles, low-ArA + ASA; open triangles, high-ArA; filled triangles, high-ArA + ASA. Values are means ± S.E.M., N=10.

free method to administer ASA. This ASA treatment also attenuated the confinement-induced cortisol response in the seabream fed the low-ArA diet, which would indicate that prostaglandins stimulated the release of cortisol at a low dietary intake of ArA. However, this would not correspond to the observed blunted cortisol response observed in seabream with a high dietary intake of ArA, which would have resulted in an elevated release of prostaglandins during stress. In fact, administration of ASA caused a slight elevation of the cortisol levels in those seabream that were supplemented with ArA in advance. This argues against the assumption that ArA-mediated effects can be attributed to the formation of COX-derived metabolites.

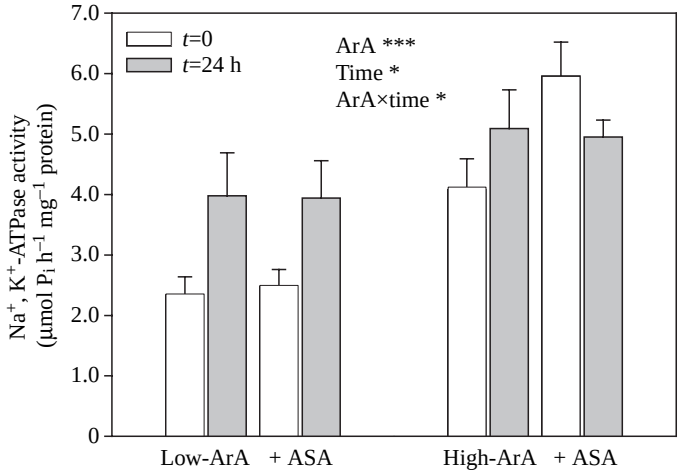

Fig. 6. Basal gill Na^+, K^+-ATPase activity (μmol P_i h^{-1} mg^{-1} protein) before (t=0 h) and 24 h after net confinement in control and ArA- and ASA-fed seabream. Samples at t=0 were collected 4 h after the last dose of ASA in the ASA-treated fish. Values are means ± S.E.M., N=10.

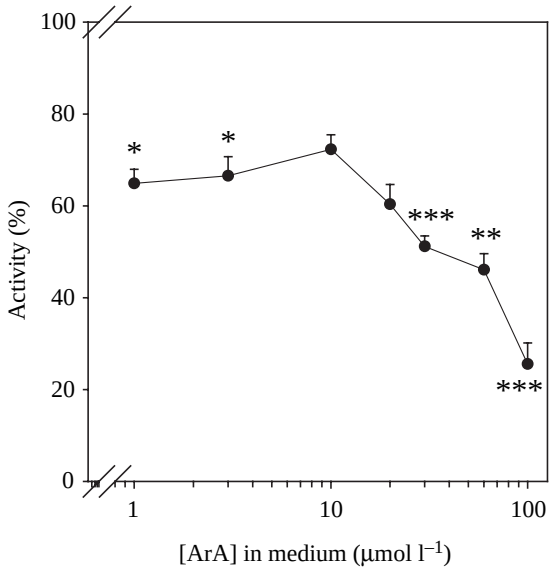

Fig. 7. The inhibition of Na^+, K^+-ATPase activity by free ArA in the incubation medium. Values are means ± S.E.M., N=10. Na^+, K^+-ATPase activity in 1% methanol was set at 100%. Activities were compared for significance to the activity at 1% methanol and 0 μmol l^{-1} ArA (see Materials and methods for details); *P<0.05, **P<0.01, ***P<0.001.

It is possible that in this study the inhibition of the COX pathway by ASA increased the availability of free ArA immediately after confinement for the alternative pathways in certain tissues. Eicosanoids resulting from conversion of ArA by lipoxygenase and epoxygenase have been shown to stimulate the release of ACTH and β-endorphin from the pituitary, as well as to modify the induced release of cortisol from the adrenals (Hirai et al., 1985). The available information on the functions of other eicosanoids besides prostaglandins in the control of the HPI axis of fish is very limited and it remains to be determined how ArA is exactly involved in this process.

Acute stress is normally associated with the release of catecholamines that promote glycogenolysis in the liver cells of fish, causing a rapid increase of plasma glucose levels (Wendelaar Bonga, 1997). The accumulation of lactate during stress either points to reduced uptake by liver cells for gluconeogenesis or to the incomplete oxidation of glucose, resulting from insufficient oxygen supply (Vijayan and Moon, 1992; Vijayan et al., 1997; Wendelaar Bonga, 1997; Fabbri et al., 1998). In the present study the dietary level of ArA had no effect on the basal levels of plasma glucose or lactate, nor did it affect the confinement-induced hyperglycemia and lactacidemia, suggesting that the catecholamine release was not affected. Nevertheless, the treatment with ASA reduced the basal glucose levels in the 'low-ArA'-fed fish, whereas ASA had no effect on the fish fed the ArA-supplemented diet. The lactate levels were affected in a different way by ASA. ASA delayed the recovery to basal levels in the low-ArA group, and augmented the lactate response in the ArA-supplemented seabream. Though it is unclear which processes were altered by the change in the dietary ArA intake, some suggestions can be made. For instance, the low ratio of n-3/n-6 PUFA might have enhanced the metabolic rate and oxygen demand, as suggested by McKenzie et al. (2000) and McKenzie (2001). On the other hand, free ArA has been shown to change the binding of catecholamines or corticosteroids to their receptors in liver cells (Vallette et al., 1991; Lee and Struve, 1992; Skalski et al., 2001), which could have reduced hepatic gluconeogenesis causing elevated lactate levels in the seabream in the present study.

Acute stress and the associated release of catecholamines can lead to an increased permeability of the branchial epithelium, leading to an influx of ions in fish in a hyperosmotic environment. This in turn stimulates the ionic extrusion by the chloride cells, located mainly in the opercula and gills (Wendelaar Bonga, 1997; McCormick, 2001). In seabream from both dietary treatments, plasma sodium and chloride levels increased within 20 min after confinement in a similar way, indicating an increased permeability. The immediate decrease in plasma potassium in all treatments was more likely caused by the enhanced uptake of K^+ into red blood

cells, in reaction to the low plasma oxygen levels associated with acute stress (Gibson et al., 2000). ArA supplementation attenuated the stressor-induced increase in plasma osmolality. The plasma levels of sodium and chloride did not exhibit a corresponding increase, suggesting the presence of other factors that influence the osmolality. Interestingly, ASA treatment appeared to counteract the effect of ArA and resulted in an increase in plasma osmolality after 1 h. Furthermore, both plasma chloride and potassium levels were enhanced by ASA in the ArA-supplemented seabream. This suggested a possible increase in the permeability of the membranes due to the combination of ASA and ArA. However, the confinement-induced increase in sodium was attenuated by ASA in that same group, arguing against an enhanced influx of ions.

Cortisol has been shown to stimulate the proliferation of branchial chloride cells and to increase the activity of ion-transporting enzymes, particularly Na^+, K^+-ATPase, the major driving force for branchial transepithelial ion transport located in the chloride cells (Wendelaar Bonga, 1997). Indeed, the branchial Na^+, K^+-ATPase activity had increased in both dietary treatments 24 h after confinement. In addition, the ArA-supplemented seabream showed a distinctly higher branchial Na^+, K^+-ATPase activity already prior to confinement. Although the effect of ASA treatment was not significant, the ArA-supplemented fish exhibited no increase in the ATPase activity when treated with ASA. From these results it is not clear whether ArA-derived prostaglandins were involved in the regulation of the branchial Na^+, K^+-ATPase. However, it also appears unlikely that the stimulating effect of ArA supplementation on the ATPase activity could be contributed to ArA itself, as the tests showed that free ArA was an inhibitor of the branchial ATPase activity *in vitro*. Inhibitory effects of unsaturated fatty acids on Na^+, K^+-ATPases have been demonstrated *in vitro* before, and were attributed to a decreased enzyme affinity for extracellular K^+ due to direct interactions of the fatty acids with sodium-pump protein subunits (Swann, 1984; Swarts et al., 1990; Haag et al., 2001). Alternatively, ArA might have stimulated the Na^+, K^+-ATPase activity in gills by enhancing the release of osmoregulatory hormones such as prolactin and thyroxin (Kolesnick et al., 1984a,b; Mustafa and Srivastava, 1989).

In this study we have demonstrated marked changes in the stress response due to feeding different dietary levels of ArA for 18 days, which emphasizes the physiological impact that a relatively small change in a single dietary fatty acid can have. Providing optimal ArA levels in commercial diets for marine species could be effective in moderating the stress response and improving survival following exposure to handling and transport, as routinely occurs during the grow-out of fish in aquaculture. Furthermore, replacing fish oils in commercial diets with vegetable oils that contain no C20 and C22 PUFA, combined with the low fatty acid elongation and desaturation activities of carnivorous/marine species, will result in reduced tissue levels of ArA, EPA and DHA (Bell, 1998; Bell and Sargent, 2003; Montero et al., 2003). It is advisable to determine the effects of such a low dietary intake of ArA and other PUFA on a longer term, focussing not only on growth performance and feed efficiency, but also on possible health effects.

List of abbreviations

ASA	acetylsalicylic acid
COX	cyclo-oxygenase
ArA	arachidonic acid (20:4n-6)
DHA	docosahexaenoic acid (22:6n-3)
EPA	eicosapentaenoic acid (20:5n-3)
PUFA	polyunsaturated fatty acids
PL	phospholipase
HPA	hypothalamus–pituitary–adrenal
HPI	hypothalamic–pituitary–interrenal
LA	linoleic acid
P_i	inorganic phosphate
PGE_2	prostaglandin E_2
α-LNA	α-linolenic acid

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References

Axelrod, J., Burch, A. and Jelsema, C. L. (1988). Receptor-mediated activation of phospholipase A_2 via GTP-binding proteins: arachidonic acid and its metabolites as second messengers. *Trends Neurosci.* **11**, 117-123.

Beckman, B. and Mustafa, T. (1992). Arachidonic acid metabolism in gill homogenate and isolated gill cells from rainbow trout, *Oncorhynchus mykiss*: the effect of osmolality, electrolytes and prolactin. *Fish Physiol. Biochem.* **10**, 213-222.

Bell, J. G. (1998). Current aspects of lipid nutrition in fish farming. In *Biology of Farmed Fish* (ed. K. D. Black and A. D. Pickering), pp. 114-145. Sheffield: Sheffield Academic Press.

Bell, J. G. and Sargent, J. R. (2003). Arachidonic acid in aquaculture feeds: current status and future opportunities. *Aquaculture* **218**, 491-499.

Bell, J. G., Castell, J. D., Tocher, D. R., Macdonald, F. M. and Sargent, J. R. (1995). Effects of different dietary arachidonic acid: docosahexaenoic acid ratios on phospholipid fatty acid compositions and prostaglandin production in juvenile turbot (*Scophthalmus maximus*). *Fish Physiol. Biochem.* **14**, 139-151.

Bessonart, M., Izquierdo, M. S., Salhi, M., Hernández-Cruz, C. M. and Fernández-Palacios, H. (1999). Effect of dietary arachidonic acid levels on growth and survival of gilthead seabream (*Sparus aurata* L.) larvae. *Aquaculture* **179**, 265-275.

Bihoreau, C., Rasolonjanahary, R., Gerozissis, K., Clauser, H. and Kordon, C. (1990). Arachidonate metabolism in the anterior pituitary: effect of arachidonate inhibitors on basal and stimulated secretion of prolactin, growth hormone and luteinizing hormone. II. Hormone release from dispersed pituitary cells. *J. Neuroendocrinol.* **2**, 445-452.

Buschbeck, M., Ghomashchi, F., Gelb, M. H., Watson, S. P. and Börsch-Haubold, A. G. (1999). Stress stimuli increase calcium-induced arachidonic acid release through phosphorylation of cytosolic phospholipase A_2. *Biochem. J.* **344**, 359-366.

Fabbri, E., Capuzzo, A. and Moon, T. W. (1998). The role of circulating

catecholamines in the regulation of fish metabolism: an overview. *Comp. Biochem. Physiol.* **120C**, 177-192.

Fountoulaki, E., Alexis, M. N., Nengas, I. and Venou, B. (2003). Effects of dietary arachidonic acid (20:4n-6), on growth, body composition, and tissue fatty acid profile of gilthead bream fingerlings (*Sparus aurata* L.). *Aquaculture* **225**, 309-323.

Gibson, J. S., Cossins, A. R. and Ellory, J. C. (2000). Oxygen-sensitive membrane transporters in vertebrate red cells. *J. Exp. Biol.* **203**, 1395-1407.

Gupta, O. P., Lahlou, B., Botella, J. and Porthé-Nibelle, J. (1985). *In vivo* and *in vitro* studies on the release of cortisol from interrenal tissue in trout. I. Effects of ACTH and prostaglandins. *Exp. Biol.* **43**, 201-212.

Haag, M., Kearns, S. D., Magada, O. N., Mphata, P. R., Claassen, N. and Kruger, M. C. (2001). Effect of arachidonic acid on duodenal enterocyte ATPases. *Prostag. Other Lipid Mediat.* **66**, 53-63.

Harel, M., Gavasso, S., Leshin, J., Gubernatis, A. and Place, A. R. (2001). The effect of tissue docosahexaenoic and arachidonic acids levels on hypersaline tolerance and leucocyte composition in striped bass (*Morone saxatilis*) larvae. *Fish Physiol. Biochem.* **24**, 113-123.

Hirai, A., Tahara, K., Tamura, Y., Saito, H., Terano, T. and Yoshida, S. (1985). Involvement of 5-lipoxygenase metabolites in ACTH-stimulated corticosteroidogenesis in rat adrenal glands. *Prostaglandins* **30**, 749-767.

Jobling, M. (2003). Do changes in Atlantic salmon, *Salmo salar* L., fillet fatty acids following a dietary switch represent wash-out or dilution? Test of a simple dilution model and its application. *Aquacult. Res.* **34**, 1215-1221.

Jobling, M. (2004). Are modifications in tissue fatty acid profiles following a change in diet the result of dilution? Test of a simple dilution model. *Aquaculture* **232**, 551-562.

Kolesnick, R. N., Musacchio, I., Thaw, C. and Gershengorn, M. C. (1984a). Arachidonic acid mobilizes calcium and stimulates prolactin secretion from GH_3 cells. *Am. J. Physiol.* **246E**, 458-462.

Kolesnick, R. N., Musacchio, I., Thaw, C. and Gershengorn, M. C. (1984b). Thyrotropin (TSH)-releasing hormone decreases phosphatidylinositol and increases unesterified arachidonic acid in thyrotropic cells: possible early events in stimulation of TSH secretion. *Endocrinology* **114**, 671-676.

Koven, W. M., Barr, Y., Lutsky, S., Ben Atia, I., Weiss, R., Harel, M., Behrens, P. and Tandler, A. (2001). The effect of dietary arachidonic acid (20:4*n*-6) on growth, survival and resistance to handling stress in gilthead seabream (*Sparus aurata*) larvae. *Aquaculture* **193**, 107-122.

Koven, W. M., Van Anholt, R. D., Lutsky, S., Ben Atia, I., Nixon, O., Ron, B. and Tandler, A. (2003). The effect of dietary arachidonic acid on growth, survival, and cortisol levels in different-age gilthead seabream larvae (*Sparus auratus*) exposed to handling or daily salinity change. *Aquaculture* **228**, 307-320.

Lands, W. E. M. (1991). Biosynthesis of prostaglandins. *Ann. Rev. Nutr.* **11**, 41-60.

Lee, P. C. and Struve, M. (1992). Unsaturated fatty acids inhibit glucocorticoid receptor binding of trout hepatic cytosol. *Comp. Biochem. Physiol.* **102B**, 707-711.

Linares, F. and Henderson, R. J. (1991). Incorporation of ^{14}C-labeled polyunsaturated fatty acids by juvenile turbot, *Scophthalmus maximus* (L.) *in vivo*. *J. Fish. Biol.* **38**, 335-347.

McCormick, S. D. (2001). Endocrine control of osmoregulation in teleost fish. *Am. Zool.* **41**, 781-794.

McKenzie, D. J. (2001). Effects of dietary fatty acids on the respiratory and cardiovascular physiology of fish. *Comp. Biochem. Physiol.* **128A**, 607-621.

McKenzie, D. J., Piraccini, G., Piccollella, M., Steffensen, J. F., Bolis, C. L. and Taylor, E. W. (2000). Effects of dietary fatty acid composition on metabolic rate and responses to hypoxia in the European eel (*Anguilla anguilla*). *Fish Physiol. Biochem.* **22**, 281-296.

Montero, D., Kalinowski, T., Obach, A., Robaina, L., Tort, L., Caballero, M. J. and Izquierdo, M. S. (2003). Vegetable lipid sources for gilthead seabream (*Sparus aurata*): effects on fish health. *Aquaculture* **225**, 353-370.

Mourente, G. and Tocher, D. R. (1994). In vivo metabolism of [1-^{14}C]linolenic acid (18:3(n-3)) and [1-^{14}C]eicosapentaenoic acid (20:5(n-3)) in a marine fish: time-course of the desaturation/elongation pathway. *Biochim. Biophys. Acta* **1212**, 109-118.

Mustafa, T. and Srivastava, K. C. (1989). Prostaglandins (eicosanoids) and their role in ectothermic organisms. *Adv. Comp. Environ. Physiol.* **5**, 157-207.

Nye, E. J., Hockings, G. I., Grice, J. E., Torpy, D. J., Walters, M. M., Crosbie, G. V., Wagenaar, M., Cooper, M. and Jackson, R. V. (1997). Aspirin inhibits vasopressin-induced hypothalamic-pituitary-adrenal activity in normal humans. *J. Clin. Endocrinol. Metab.* **82**, 812-817.

Robin, J. H., Regost, C., Arzel, J. and Kaushik, S. (2003). Fatty acid profile of fish following a change in dietary fatty acid source: model of fatty acid composition with a dilution hypothesis. *Aquaculture* **225**, 283-293.

Rowley, A. F., Knight, J., Lloyd-Evans, P., Holland, J. W. and Vickers, P. J. (1995). Eicosanoids and their role in immune modulation in fish – a brief overview. *Fish Shellfish Immun.* **5**, 549-567.

Skalski, M., Goto, M., Ravindranath, T., Myers, T. and Zeller, W. P. (2001). Omega-3 polyunsaturated fatty acid enriched diet attenuates stress-induced lactacidemia in 10-day-old rats. *Pediatr. Int.* **43**, 409-416.

Smith, W. L. (1989). The eicosanoids and their biochemical mechanisms of action. *Biochem. J.* **259**, 315-324.

Swann, A. C. (1984). Free fatty acids and (Na^+, K^+)-ATPase: effects on cation regulation, enzyme conformation, and interactions with ethanol. *Arch. Biochem. Biophys.* **233**, 354-361.

Swarts, H. G. P., Schuurmans Steckhoven, F. M. A. H. and De Pont, J. J. H. H. M. (1990). Binding of unsaturated fatty acids to Na^+, K^+-ATPase leading to inhibition and inactivation. *Biochim. Biophys. Acta* **1024**, 32-40.

Tocher, D. R. and Ghioni, C. (1999). Fatty acid metabolism in marine fish: low activity of fatty acyl Δ5-desaturation in gilthead seabream (*Sparus aurata*) cells. *Lipids* **34**, 433-440.

Vallette, G., Vanet, A., Sumida, C. and Nunez, E. A. (1991). Modulatory effects of unsaturated fatty acids on the binding of glucocorticoids to rat liver glucocorticoid receptors. *Endocrinology* **129**, 1363-1369.

Van Anholt, R. D., Spanings, T., Koven, W. M. and Wendelaar Bonga, S. E. (2003). Effects of acetylsalicylic acid on thyroid hormones, prolactins, and the stress response of tilapia (*Oreochromis mossambicus*). *Am. J. Physiol.* **285**, R1098-R1106.

Van Anholt, R. D., Koven, W. M., Lutzky, S. and Wendelaar Bonga, S. E. (2004). Dietary supplementation with arachidonic acid alters the stress response of gilthead seabream (*Sparus aurata*) larvae. *Aquaculture* (in press).

Vijayan, M. M. and Moon, T. W. (1992). Acute handling stress alters hepatic glycogen metabolism in food-deprived rainbow trout (*Oncorhynchus mykiss*). *Can. J. Fish. Aquat. Sci.* **49**, 2260-2266.

Vijayan, M. M., Pereira, C., Grau, E. G. and Iwama, G. K. (1997). Metabolic responses associated with confinement stress in tilapia: the role of cortisol. *Comp. Biochem. Physiol.* **116C**, 89-95.

Wales, N. A. M. (1988). Hormone studies in *Myxine glutinosa*: effects of the eicosanoids arachidonic acid, prostaglandin E_1, E_2, A_2, $F_{2\alpha}$, thromboxane B_2 and of indomethacin on plasma cortisol, blood pressure, urine flow and electrolyte balance. *J. Comp. Physiol.* **158B**, 621-626.

Wang, H., Walker, S. W., Mason, J. I., Morley, S. D. and Williams, B. C. (2000). Role of arachidonic acid metabolism in ACTH-stimulated cortisol secretion by bovine adrenocortical cells. *Endocrinol. Res.* **26**, 705-709.

Wendelaar Bonga, S. E. (1997). The stress response in fish. *Physiol. Rev.* **77**, 591-625.

Zacharieva, S., Borissova, A. M., Andonova, K., Stoeva, I. and Matrozov, P. (1992). Role of Prostaglandin E_2 (PGE_2) on the corticotropin-releasing hormone (CRH)-induced ACTH release in healthy men. *Horm. Metab. Res.* **24**, 336-338.