Effects of long-term dietary exposure to low levels of dioxins on growth and biochemical responses in juvenile gilthead seabream 'sparus aurata'

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1. Introduction

It is generally accepted that PCDD/Fs enter the food chain, diet being the main source of human exposure to these contaminants, accounting for up to 98% of the total intake[1]. In particular, fish and seafood products may account up to a third of the total human intake[2]. Recent dioxin contamination episodes have demonstrated how these contaminants could easily reach the top of the trophic chain, up to humans. For that reason, several surveillance programs have been undertaken with the aim to get about a better insight of behaviour and trends of PCDD/Fs. These have also resulted in the enforcement of legal measures to reduce the risks derived from exposure to these pollutants. A large number of studies have reported valuable data about the concentrations of dioxins and '*dioxin-like*' compounds in food and feed, temporal trends and the estimated daily intake in humans. Nevertheless, at this moment little information is available on the mechanisms of transfer of these pollutants through the food web.

Research has been carried out on bioaccumulation, distribution and kinetics of PCDD/Fs in food products. In particular, several studies are focused on pigs and chickens after being exposed to these pollutants through contaminated feed[3,4]. Dietary exposure to environmentally realistic low doses of dioxins and furans in long-term studies might be particularly interesting in aquaculture as the dioxin levels reached, at the time the fish attain the limit commercial size may compromise the quality of the final product. Dioxin exposure has seen to negatively affect fish reproduction, either in laboratory experiments or in larger scale studies, where it has been associated to fish mass mortality events[5]. Thus, in this study we attempted to reproduce similar rearing conditions similar to those usually found in hatcheries with the aim of evaluating the effects of a dioxin exposition. To this end, dioxins were used as a supplement in the ongrowing diet of gilthead seabream (*Sparus aurata*) in order to follow up the bioaccumulation of these toxicants in muscle (carcasses) and liver and to observe the effects on the growth, survival and behaviour of the fish.

In addition to the bioaccumulation study, a second objective of the present work was to evaluate the toxicological effects due to dioxin exposure. Toxicity mediated action of PCDD/Fs occurs through the Ah receptor and determines induction or CYP1A gene/protein and its specific ethoxyresorufin O-deethylase (EROD) activity as part of phase I metabolism. Effects on the conjugation enzymes (phase II) have also been described although in a much less pronounced manner[6]. Effects on antioxidant enzymes could also be expected as oxyradical formation is a by-product of phase I metabolism.

2. Materials and Methods

Gilthead seabream juveniles (15.58±3.72 g initial weight) were stocked during a year in four 1500 l tanks (50 juveniles per tank) at CA-IRTA under the following conditions: temperature 18±1ºC, salinity 35±1 ppt, oxygen 6±1 mg/l and photoperiod 12hL: 12hD. The fish were fed by hand daily, *ad libitum,* using a commercial diet for seabream with increasing pellet diameter according to the weight of the fish. In two of the tanks (experimental tanks) the fish were fed with the dry feed coated with olive oil (5 g per kg of feed) in which a known amount of all seventeen 2,3,7,8 chlorosubstituted PCDD/Fs was added (5 ng WHO-TEQ/mL of oil), meanwhile the other two tanks were considered as control and fed with the same kind of pellets coated with equal amount of the non-spiked olive oil. Every month, 10 fish per tank were sampled for weight gain control. Growth was calculated and expressed as monthly weight gain per fish (g/fish), weight gain (w_f-w_i/w_i * 100) and specific growth rate (SGR, % bw, day^{-1}). In addition, daily food intake

and quantity of oil used in pellet preparation were recorded in order to calculate the feed intake (g feed/body weight), the food conversion ratio (FCR = food eaten / weight gained), and the quantity of food and oil ingested per fish.

Several samplings were also undertaken in order to obtain samples for dioxin analysis. For each of the campaigns, at least 5 fish per tank were sacrificed with an overdose of anaesthetic or with ice, depending on the type of sampling, and livers were dissected out from the carcasses for a separate dioxin bioaccumulation analysis. When a different kind of pellet was used, a sample of dry feed was also taken and analysed. Once collected, samples were inmediatly sent to the laboratory and stored at -20 ºC until analysis. Dioxin analyses were carried out following classical procedures reported elsewhere[7]. Briefly, the samples were freeze-dried (carcasses and livers) and homogenized prior to the analysis. Next, they were spiked with known amounts of a mixture of $^{13}C_{12}$-PCDD/PCDFs and then extracted in a Soxhlet for 24h with toluene:cyclohexane (1:1). The extracts were rotary evaporated and redissolved in 100 mL n-hexane. Organic components, fat and other interfering substances were removed by treating the n-hexane extracts with sulphuric acid. The extracts were then rotary concentrated and filtered prior to the clean up process carried out with an automated Power Prep^{TM} system (FMS Inc., MA). Finally, instrumental analyses were performed by HRGC/HRMS on a Agilent gas chromatograph coupled to a Micromass Ultima NT high resolution mass spectrometer (EBE geometry) controlled by a Masslynx data system, using a positive electron ionization (EI+) source and operating in the SIM mode. The chromatographic column used was a DB-5ms (40m x 0.18 mm i.d. x 0.18µm film thickness) from J&W Scientific (CA, USA). Quantification was carried out by the isotopic dilution method.

To evaluate biotransformation enzymes, fish liver homogenates were prepared using a 100 mM buffer phosphate containing (1 mM EDTA, 0.1 mM PMSF and 1mM DTT) in a liver:buffer (1:4) ratio. After centrifugation to 10,000 g x 20 min the supernatant obtained (S9 fraction) was used for biochemical determinations (n=10) in both control and exposed fish. The antioxidants catalase and total glutathione peroxidase (t-GPX), EROD and GST activities as well as lipid peroxidation (LP) measured as malonaldehide (MDA) equivalents were all determined in this fraction as described elsewhere[8].

3. Results and discussion

The initial design of the experiment for the bioaccumulation study was based on at least 6 different sampling campaigns along one year, including the background levels (t_0), in parallel to the increase in pellet diameter of feed. Preliminary results from the three first campaigns are presented here. Table 1 summarizes the results of dioxin content in fish carcass and liver, expressed in pg WHO-TEQ/g fresh weight (f.w). Significant bioaccumulation was achieved earlier by those fish exposed to the contaminants, the levels in carcasses in the second campaign being approximately 20-25 folds higher with respect to the background level. The values remained constant within the third campaign, even though this fact must be confirmed with data from future campaigns. Non-exposed animals showed similar low values along the whole period considered, with concentrations in the range of 0.10 to 0.23 pg WHO-TEQ/g f.w. of the carcass. In general, the same trend was observed in the case of the liver although the levels detected were significantly higher due to the larger accumulation that takes place in this organ.

Table 1. Results of concentrations of PCDD/Fs expressed in pg WHO-TEQ/g f.w. (upperbound) in liver and carcasses of control and exposed fish. Mean values and SD of the two tanks are reported in each case.

	Carcasses Control	Carcasses Exposed	Liver Control	Liver Exposed
Background	0.230	-	0.610	-
2nd Campaign	0.133±0.021	5.503±0.123	0.492±0.185	8.451±0.456
3rdCampaign	0.099±0.005	4.389±0.034	0.832±0.601	7.616±1.270

The profile analysis of dioxins reproduced in Figure 1 shows that the control group presented a typical fish congener-specific distribution, similar to that found in the background animals and characterized by a predominant presence of the low chlorinated congeners 2,3,7,8-TCDF and 2,3,4,7,8-PeCDF. On the other hand a different profile was observed in the exposed group of fish, which strongly reflected the influence of dioxin congener-distribution in the spiked oil added to the feed. In this sense, the fish fed dioxin-enriched feed seemed to have a preferential accumulation of the lowest chlorinated congeners, tetra- and penta- even though feed was spiked with same amount of penta-, hexa- and hepta chlorinated compounds and double the amount of octa-chlorinated substances (Figure 1).

Table 2 shows the growth of the fish and the results of feeding. From a physiological point of view, no significant differences were detected between control and exposed groups along the rearing period. Fish behaviour in terms of feeding, swimming and /or aggression was the same in all the tanks, and no mortalities were recorded. Weight gain was similar between the two groups, despite growth in December being lower than in the other months, especially for the exposed group. Feed intake was between 0.16 and 0.34% body weight in all the tanks without significant differences among the groups. Conversion rate (FCR) was close to 1 in all the cases except for the data of December, due to the lower growth of the fish registered in that month. In addition, feed and oil intake per fish were similar among the groups.

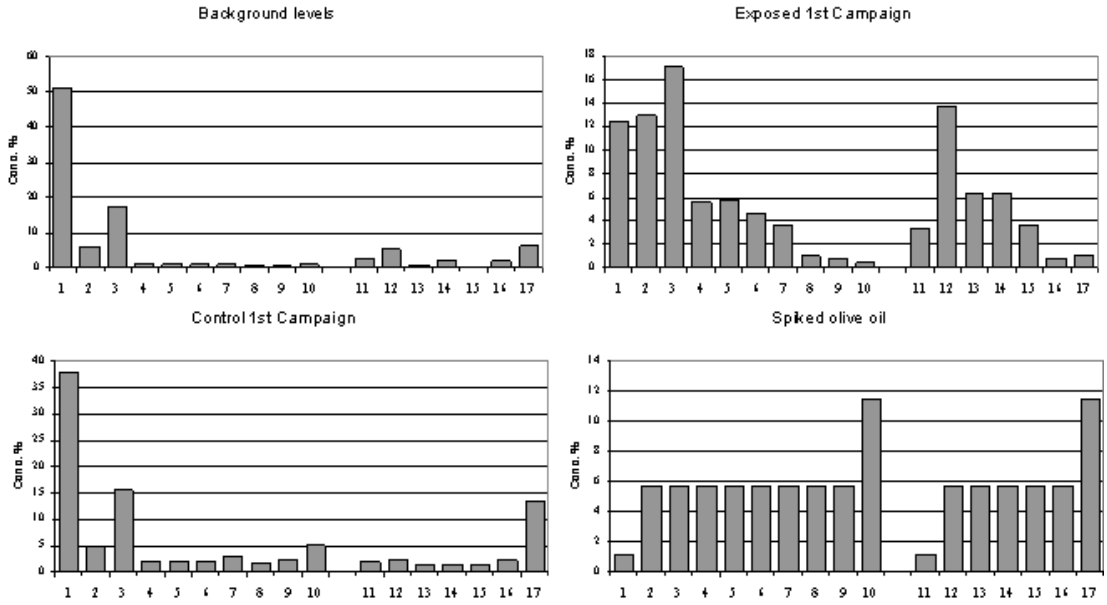

Figure1. Comparison of 2,3,7,8 PCDD/Fs in background, control, exposed fish carcasses as well as in spiked oil. 1: 2,3,7,8-TCDF; 2: 1,2,3,7,8-PeCDF; 3: 2,3,4,7,8-PeCDF; 4: 1,2,3,4,7,8-HxCDF; 5: 1,2,3,6,7,8-HxCDF; 6: 2,3,4,6,7,8-HxCDF; 7: 1,2,3,7,8,9-HxCDF; 8: 1,2,3,4,6,7,8-HpCDF; 9: 1,2,3,4,7,8,9-HpCDF; 10: OCDF; 11: 2,3,7,8-TCDD; 12: 1,2,3,7,8-PeCDD; 13: 1,2,3,4,7,8-HxCDD; 14: 1,2,3,6,7,8-HxCDD; 15: 1,2,3,7,8,9-HxCDD; 16: 1,2,3,4,6,7,8-HpCDD; 17: OCDD

Table 2. Growth and feeding of control (C) and dioxin (D) treated groups

Month	Average weight (g)		Weight gain (%)		SGR (%bw day^{-1})		Feed intake (g feed/fish)		Feed intake (%bw)		FCR		Oil intake (g oil/fish)	
	C	**D**	**C**	**D**	**C**	**D**	**C**	**D**	**C**	**D**	**C**	**D**	**C**	**D**
Jun	21.9	21.9	40.6	41.0	1.22	1.22	6.8	6.6	0.30	0.31	1.08	1.03	0.034	0.033
Jul	34.9	34.2	59.1	55.7	1.68	1.60	10.7	10.9	0.32	0.31	0.83	0.89	0.054	0.055
Aug	52.2	54.9	49.9	60.5	1.35	1.51	16.4	18.3	0.33	0.31	0.95	0.88	0.082	0.092
Sep	77.7	79.5	48.7	44.8	1.33	1.24	25.0	27.2	0.34	0.32	0.98	1.11	0.125	0.136
Oct	104.6	115.1	34.7	44.8	0.93	1.16	30.8	35.5	0.31	0.29	1.14	1.00	0.154	0.178
Nov	128.9	148.1	23.3	28.7	0.70	0.84	35.4	35.6	0.24	0.27	1.46	1.08	0.177	0.178
Dec	144.4	152.4	**12.0**	**2.9**	**0.46**	**0.11**	36.3	35.2	0.23	0.25	**2.34**	**8.19**	0.182	0.176
Jan	190.2	202.3	31.7	32.8	0.77	0.79	44.8	49.1	0.24	0.23	0.98	0.98	0.224	0.245
Feb	227.4	244.6	19.6	20.9	0.51	0.54	47.0	53.5	0.22	0.21	1.26	1.26	0.235	0.268
Mar	270.8	286.1	19.1	16.9	0.76	0.68	43.4	46.5	0.16	0.16	1.00	1.12	0.217	0.233

SGR = 100 * (e^G-1), being G = Ln (w_f/w_i)/ t_f-t_i and w_f, w_i, t_f, t_i the final and initial weight (w) and final and initial time (t). FCR = food eaten / weight gained.

The results corresponding to biomarkers of dioxin-exposure or effect are presented in Table 3. Dietary

exposure of PCDD/Fs had no effect in the antioxidant enzymes catalase and t-GPX, neither in oxidative damage occurring to lipids (LP). Nevertheless, EROD activity was significantly enhanced in the dioxin exposed fish ($p<0.05$; one-way ANOVA) as well as GST activity although in this case it was not statistically significant. Dietary exposure of 2,3,7,8-TCDD at doses ranging from 40 to 400 pg/g feed enhanced EROD in two fish species (*Oncorhynchus mykiss* and *Coregonus clupeaformis*) the threshold necessary to induce this response being of 15 and 45 pg/g, respectively[9]. Responses of other biotransformation enzymes such as GST are not so evident and despite an 83-fold induction of EROD in dioxin-exposed carp (*Carassius auratus gibelio*) the GST elevation, albeit significant, was only 1.4-fold[6]. Our observations point to EROD as a sensitive biomarker of dioxin exposure but species sensitivity is a factor that must be consider. Other phase II enzymes such as UDPGT or the antioxidant DT-diaphorase, which are Ah mediated, might be other biomarkers likely to respond to dioxins. Overall, it seems that the enhanced metabolism due to dioxin exposure is not responsible for oxidative stress or any other apparent decrease in fitness in the treated fish.

Table 3. Results of biomarkers of dioxin-exposure.

	Catalase (mmol/min/mgprot)	**t-GPX** (nmol/min/mg prot)	**GST** (nmol/min/mg prot)	**EROD** (pmol/min/mg prot)	LP (nmol MDA/g w.w)
Control (n = 10)	553 ± 76.4	200 ± 20.4	877 ± 159	58 ± 9.1	8.4 ± 1.0
Exposed (n = 10)	461 ± 54.3	187 ± 12.5	1383 ± 125	103 ± 14.8	7.1 ± 1.3

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