Environmental doses of perfluorooctanoic acid change the expression of genes in target tissues of common carp

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***Abbreviations*:** PFOA, perfluorooctanoic acid; GST, glutathione S-transferase; RT-qPCR, real-time quantitative PCR; LOD, limit of detection; HPG, hypothalamic-pituitary-gonadal.

Abstract

We aimed to evaluate the effects of environmental doses of perfluorooctanoic acid (PFOA) on bioconcentration and gene expression in common carp (*Cyprinus carpio*). Adult male and female carp were exposed to environmental (200 ng/L) and experimental (2 mg/L) doses of PFOA for 56 d. Carp exposed to 200 ng/L had levels of PFOA below the level of detection in all tissue samples analyzed, whereas variable concentrations were measurable in various tissues from carp exposed to 2 mg/L. The expression level of the glutathione S-transferase (GST) gene, coding for a detoxifying enzyme, increased in a PFOA dose- dependent manner in liver tissues from 200 ng/L to 2 mg/L exposure ($p < 0.05$). The expression levels of CYP19A gene, coding for the enzyme that converts testosterone into estrogen, were altered in gonadal tissues from male and female carp exposed to either 200 ng/L or 2 mg/L; expression increased in male gonads and decreased in female gonads. Unexpectedly, the expression levels of CYP19A in male and female gonads from carp exposed to 200 ng/L or 2 mg/L were similar ($p > 0.05$). Therefore, even though environmental doses of PFOA did not accumulate in tissues of the common carp, they did affect the gene expression levels of GST in the liver and CYP19A in the gonads. These observations raise concerns that exposure to environmental doses of PFOA may affect gene expression in animals and possibly in humans, with important health consequences.

1. Introduction

Perfluorooctanoic acid (PFOA) is a synthetic molecule of perfluorinated compounds [1]. It is widely used as a surfactant in industrial manufacturing of lubricants, medical equipment, paper and textile coatings, upholstery, shiny food packaging, and fire-fighting foams. Perfluorooctanoic acid is highly soluble in water and is nonbiodegradable. Various concentrations of PFOA have been detected in soil, air, and aquatic environments worldwide, indicating that PFOA is a persistent synthetic pollutant [2]. Water is the major environmental reservoir of PFOA, as well as the main source of exposure for animals and humans [3]. Rivers and lakes with direct industrial emissions contain ng/L to mg/L concentrations of PFOA, whereas ocean levels are in the range of 10 to 100 pg/L [2].

Concentrations of PFOA, ranging from ng/L to mg/L, have been detected also in urban wastewater and in bottled water for human consumption [4].

There is growing concern that environmental accumulation of PFOA is a risk factor for animal and human health. Biomonitoring studies have revealed the presence of PFOA in wildlife tissues [5,6] and in blood/serum of various human popula- tions [7], suggesting that PFOA is a bioaccumulative compound. Bioaccumulation and toxic effects on the liver, kidneys, and gonads, as well as carcinogenic activity in the liver, have been revealed in fish and mammals exposed to PFOA [8–18]. Moreover, changes in expression of genes, such as the glutathione S-transferase (GST), which encodes a detoxifying enzyme, and CYP19A, which encodes the aromatase enzyme involved in steroidogenesis, also were reported in the liver and gonads [19,20]. However, most of these studies have been performed with experimental doses of PFOA that are higher than those detected in the natural environment of animals, and much higher than those to which humans are usually ex- posed [9,12,13,21]. These findings have raised the question of whether environmental concentrations of PFOA affect the health of living beings.

Therefore, in the present study, we investigated the effects of environmental concentrations of PFOA on gene expression levels in target tissues of common carp, Cyprinus carpio. Adult male and female carp were exposed for 56 d to the environmental dose of 200 ng/L of PFOA, which is an intermediate concentration in the range of those measured at the upstream reaches of the Po River, between Turin and Milan, in northern Italy [22,23]. Bioaccumulation of PFOA in tissues and mRNA expression levels of GST and CYP19A genes were analyzed in 2 PFOA target tissues: the liver and gonads. The results were compared with those obtained from carp exposed to the experimental dose of 2 mg/L of PFOA.

2. Materials and methods

2.1. Fish exposure test and tissue collection

Thirty 2-yr-old carp were purchased from a local fish farm and acclimated according to Test No. 305 of the Organisation for Economic Co-operation and Development (1996). The captive maintenance

procedures and research protocols were approved by the University of Ferrara Institutional Animal Care and Use Committee and by the Italian Ministry of Health (Law 116 of 27 January 1992) and the European Union (Directive 86/609/EEC of the European Union Parliament and of the Council of 24 November 1986). Four weeks before starting the experiment, the carp were equally divided into 3 different test tanks (10 fish per tank) and left to adapt to experimental conditions. The carp were fed with a commercial pellet food (Tetra Pond Pellets Mini) 3 times per week at 2% of their total body weight. Waste and uneaten food were removed regularly. The carp had an average total length of 19.3 ± 2.5 cm and weight of 104.8 ± 27.8 g (mean ± standard deviation [SD]). A flow-through exposure test was conducted for 56 d by means of a system that continuously delivered PFOA, diluted to the test concentrations, to the test tanks. The tanks were 120-L glass aquaria filled with tap water: a continuous supply of fresh water was provided at a flow-through rate of 500 mL/min. Exposures were carried out in 2 different tanks containing 200 ng/L of PFOA or 2 mg/L of PFOA; a third tank was used as negative control. These concentrations were selected on the basis of environmental reports [22,23] and experimental data from the literature [12,24,25]. The stock solutions were prepared by dissolving the PFOA (chemical purity 96%; Sigma-Aldrich) in distilled water and then delivering it into the 2 treatment tanks by a peristaltic pump at a flow rate of 0.42 mL/min. At time 0 of the exposure, an initial volume of the stock solution was added to the treatments tanks to immediately achieve the desired exposure concentration. The actual concentration of PFOA in the test water from each test tank was measured by liquid chromatography–mass spectrometry (LC–MS) 3 times during the exposure period (Table 1). The results of these analyses indicated that PFOA concentration was maintained at > 80% of each nominal concentration. The water temperature, pH, and oxygen saturation were recorded 3 times a week and kept at 10 to 15 8C, 6.70 to 8.00, and above 80%, respectively. At the end of the 56-d exposure, the fish were anesthetized with MS-222, killed by a deep cut through the neck, dissected, and sexed. The sex ratio was 1:1. Blood (0.5 mL), liver, and gonad (1 g from each carp) were flash-frozen and stored at –20 8C until they were analyzed for PFOA.

109 *2.2. Analysis of PFOA in tissues*

110 Concentrations of PFOA in tissues were analyzed to determine the accumulation after 56 d of
111 waterborne exposure. Perfluorooctanoic acid was extracted by use of an ion-pairing extraction
112 procedure and measured by use of high-performance liquid chromatography (HPLC) with
113 electrospray ionization tandem mass spectrometry, according to a widely used method [6,26–28].
114 Approximately 1 g or 1 mL of sample was homogenized with 5 mL of Ultrapure water supplied by a
115 Milli-Q system from Millipore. One milliliter of 0.5 M of tetrabutylammonium hydrogen sulphate
116 (Sigma-Aldrich) solution and 2 mL of sodium carbonate buffer (Sigma-Aldrich; 0.25 M, pH 10) were
117 added to 1 mL of the homogenate samples in a polypropylene tube and thoroughly mixed before
118 extraction. Five milliliters of methyl tert-butyl ether (MTBE; Sigma-Aldrich) were added to the above
119 mixture and shaken for 20 min. The organic and aqueous layers were separated by centrifugation, and
120 an exact volume of MTBE (4 mL) was removed from the solution. The aqueous mixture was rinsed
121 with MTBE and separated twice; both the rinses were combined in a second polypropylene tube.
122 The solvent was evaporated under nitrogen and replaced with 0.5 mL of HPLC grade methanol
123 (Sigma-Aldrich). This extract was passed through a nylon filter (0.2 mm; Supelco) into an HPLC
124 vial. Extraction blanks were prepared using Milli-Q water. Analytes' separation was performed using
125 a Finnigan Surveyor Plus HPLC System consisting of a quaternary pump, vacuum degasser, and
126 autosampler. Chromatographic separation was achieved with a Betasil C18 column (50 x 2.1-mm i.d.,
127 5 lm) supplied by Thermo Electron. For quantitative determination, the HPLC system was interfaced
128 to a Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron) operating in negative
129 electrospray mode. Primary and product ions monitored for PFOA determination were 498.8 >
130 368.9. Ten microliters of each extract were injected in the LC–MS with 2mM LC–MS grade
131 ammonium acetate (> 99%), as the mobile phase starting at 10% methanol (HPLC grade, Sigma-
132 Aldrich). At a flow rate of 300 mL/min, the gradient increased to 95% methanol at 10 min before
133 reverting to original conditions at 15 min. Column temperature was maintained at 25 °C. Standards
134 for the 5-point calibration curve were prepared by progressive dilution with methanol from a neat
135 standard purchased from Dr. Ehrenstorfer (Augsburg, Germany), and concentrations were evaluated

in comparison with this unex- tracted standard curve and were not corrected for the recoveries or for the purity of the standards (more than 98%). Individual stock solutions of the target analytes were prepared in methanol and stored in polypropylene bottles at –20 8C. Limit of detection (LOD), determined as 3 times the signal-to-noise ratio, was 0.4 ng/g wet weight. Chemicals and reagents were analytical grade, and Teflon-coated labware was avoided during the entire process of sampling, pretreatment, and analysis to minimize contamination of the samples. Data quality assurance and quality control protocols included matrix spikes, laboratory blanks, and continuing calibration verification. Blanks were analyzed with each set of 5 tissue samples as a check for possible laboratory contamination and interferences; recoveries, as- sessed using spiked matrix with a concentration of 5 ng/g of the analyte, were more than 93% in blood, and more than 89% in solid tissues (gonad and liver).

2.3. RNA extraction

Total RNA was isolated from 20 to 30 mg of liver and gonadal tissues by use of an RNeasy Mini Kit (Qiagen) and treated with RNeasy-free DNase I (Qiagen) according to the manufacturer's instructions [29]. Purity of RNA was determined by measuring the A260/A280 absorbance ratio using a NanoDrop 2000c (Thermo Fisher Scientific). Quality of RNA was checked by agarose gel (1.2%) electrophoresis.

2.4. cDNA preparation

Five hundred nanograms of RNA from each sample were reverse transcribed with a cDNA synthesis kit by use of random primers (ImProm-II Reverse Transcription System; Promega), adhering to the manufacturer's instructions. The cDNA products were subsequently used as templates for real-time quantitative polymerase chain reaction (RT-qPCR) reactions.

2.5. Real-time quantitative PCR

Real-time quantitative PCR for CYP19A and GST expression analysis was carried out using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich). The primers used for CYP19A expression [30] were 5'-GGTTTGCATCACTTCCACAA-3'- for 5'-AATACGGTCTGCCAGGTGTC-3'-rev [31]. The size of the PCR product was 198 bp (position 546-762 of the C. carpio CYP19A mRNA. Gene Bank accession number DQ534411). The primers used for GST expression were 5'-CTATGTTAAGG- CATTTGGGTCGCAAAC-3'- for 5'-ATCCACATAGCTCTTGA-GAGTTGGGAAGG-3'-rev [32]. The size of the PCR product was 338 bp (position 203-540 of the C. carpio Pi-class GST mRNA. Gene Bank accession number DQ411313). The glyceraldehyde 3-phosphate dehydrogenase gene was used as an internal control for gene expression analysis. The primers used were 5'-AGGGGCTCAGTATGTTGTGG-3'- for, and 5'-AGGAGGCATTGCTGACAACT-3'-rev [31]. The size of the PCR product was 185 bp (position 278-422. Gene Bank accession number AJ870982.1). Cycling parameters for all reactions were 96 8C for 10 min, 45 repeats at 95 8C for 30 s, and 62 8C for 1 min. The specificities of the PCR products were evaluated by melting curve profiles (temperature range from 60–95°C), with temperature increasing at a rate of 0.5 8C s-1 and by 2% agarose gel electrophoresis. Data were analyzed by use of the 2-DDCt method [33]. Glutathione S-transferase mRNA and CYP19A mRNA were measured individually in each liver and gonadal tissue, respectively. Then fold-change values were averaged in each group. All RT-qPCR assays were performed 3 times (each with 3 repeated measures) and results were averaged. To detect possible contamination, negative control reactions without cDNA templates were included.

2.6. Statistics

The statistical package Statistica Ver 7.1 (StatSoft) was used to analyze all data, with significance set at the 0.05 level. The mean, standard error, and range were determined for PFOA in tissues of each carp group. To allow inclusion of all samples in the statistical analyses, measured concentrations below the detection limit (0.4 ng g-1 wet wt) were assigned a value of one- half the LOD. Shapiro–Wilk and Levene's tests were used to check the normality and the homogeneity of data variance. One-

189 way analysis of variance and the Student's t test were used to compare PFOA tissue concentrations
190 among the treatment groups and between sexes.
191
192 **3. Results**
193 No mortality was observed during the acclimation or test in either control or tested fish. No carp
194 showed signs of clinical disease and abnormality such as erratic swimming, incoordina- tion of
195 movements, respiratory distress, or hyporeflexia.
196
197 *3.1. PFOA concentrations in tissue samples*
198 In all tissue samples from unexposed carp and from carp exposed to the environmental dose of 200
199 ng/L of PFOA, the PFOA concentrations were below the LOD and significantly lower than those in
200 carp exposed to the the experimental dose of 2 mg/L ($p < 0.05$). Mean PFOA concentrations from
201 carp exposed to the experimental dose of 2 mg/L ranged from 4 to 65 ng g-1 wet weight (Table 2).
202 Levels of PFOA were highest in blood, and then in liver and gonads. The mean PFOA concentration
203 in blood was significantly higher than that in other tissues ($p < 0.05$; Table 2). There were no
204 significant differences in mean tissue PFOA concentrations between males and females ($p > 0.05$;
205 Table 2).
206
207 *3.2. Expression level of GST mRNA in liver tissue*
208 Real-time quantitative PCR analysis of all liver tissues from carp (males and females together)
209 indicated that mRNA expression of GST was significantly higher in the environmental dose of
210 200 ng/L PFOA- and the experimental dose of 2 mg/L PFOA-treated groups than in unexposed carp
211 (Figure 1). Specifically, the GST mRNA levels were 2.3-fold higher in livers from carp exposed to the
212 environmental dose of 200 ng/L of PFOA and 5.4-fold higher in livers from carp exposed to the
213 experimental dose of 2 mg/L of PFOA than in unexposed carp (Figure 1). There were no differences
214 in liver GST expression level between males and females (data not shown).
215

3.3. Expression level of CYP19A mRNA in gonads

Expression levels of CYP19A mRNA in testes (n = 5) and ovaries (n = 5) from carp exposed to the environmental dose of 200 ng/L or the experimental dose of 2 mg/L of PFOA were analyzed with RT-qPCR and compared with corresponding unexposed carp (n = 5 males and n = 5 females). The expres- sion levels of CYP19A mRNA were 2.2-fold higher in testes from carp exposed to the environmental dose of 200 ng/L of PFOA and 3.4-fold higher in testes exposed to the experimen- tal dose of 2 mg/L of PFOA than in testes from unexposed carp (Figure 2). In ovaries, CYP19A expression was significantly downregulated by 3.7-fold in female carp exposed to the environmental dose of 200 ng/L of PFOA and by 3.4-fold in carp exposed to the experimental dose of 2 mg/L of PFOA compared with expression in ovaries from unexposed carp (Figure 3). Differences in CYP19A mRNA expression levels were no different between carp exposed to the environmental dose of 200 ng/L and the experimental dose of 2 mg/L of PFOA ($p > 0.05$).

4. Discussion

The present study reports—for the first time that we know of—the effects of environmental doses of PFOA in adult male and female carp. Perfluorooctanoic acid has been found in the aqueous environment worldwide at several ng/L concentrations as reported by Suja et al. [1] with reference to surface and tap water samples collected from different countries. The levels of PFOA measured in the control tank (Table 1) were similar to those reported in many surface waters [1], whereas the environmental dose of 200 ng/L used in the present study represents a high level of contamination reached in impacted rivers such as the Po River in Italy [22,23] or Cape Fear River (Wilmington, NC, USA) [34]. Thus PFOA effects and impact on fish biomarkers were herein evaluated under a field-relevant exposure scenario.

We investigated the accumulation of PFOA in carp tissues and changes in expression levels of the GST gene in the liver and the CYP19A gene in gonads, which are the main target tissues of PFOA. Our results showed that carp exposed for 56 d to the environmental dose of 200 ng/L of PFOA had levels of PFOA below the LOD in all tissue samples analyzed. Conversely, and in agreement with

243 previously reported data [17], concentrations of PFOA were detected in various tissues from carp
244 exposed to the experimental dose of 2 mg/L of PFOA, with the highest levels in blood.
245 Perfluorooctanoic acid has low bioconcentration factors (BCFs) in fish (Organisation for Economic
246 Co-operation and Development [35]); a BCF between 3.1 and 9.4 was reported for carp [36,37]. Thus
247 it is not surprising that PFOA was not measurable in carp exposed to the lowest concentration
248 (200 ng/L) and with the LOD of the method used in the present study.
249 Importantly, even though PFOA at environmental concen- trations did not accumulate in the liver,
250 liver tissues from carp exposed to the environmental dose of 200 ng/L of PFOA expressed GST
251 mRNA levels significantly higher than those from unexposed carp. Moreover, GST mRNA levels in
252 liver tissues from carp exposed to the experimental dose of 2 mg/L of PFOA were significantly higher
253 than those from unexposed carp as well as those from carp exposed to the environmental dose of
254 200 ng/L PFOA. Although only 2 PFOA concentrations were used in our experiments, this result
255 suggests that the expression level of GST may be regulated in a PFOA dose-dependent manner.
256 Because GST expression is induced by increased levels of oxidant molecules [38], our data are
257 consistent with PFOA acting as an inducer of reactive oxygen species in carp liver, as previously
258 suggested in other animal models [39]. However, other studies of fish and mammals reported reduced
259 expression of GST after administration/exposure to PFOA, indicating that PFOA can act differently in
260 liver of some species.
261 In the present study, male gonads from carp exposed to the environmental dose of 200 ng/L of
262 PFOA showed CYP19A mRNA levels significantly higher than those from unexposed male carp. In
263 contrast, the expression levels of CYP19A were significantly lower in gonads from female carp
264 exposed to the environmental dose of 200 ng/L of PFOA than those from unexposed female carp.
265 It is well known that aromatase is the enzyme that converts testosterone into estrogen and plays a key
266 role in the establishment and maintenance of sex in fishes. Almost absent in male gonads, CYP19A is
267 highly expressed in female gonads. In addition, certain environmental contami- nants, including
268 PFOA, act as endocrine disruptors by modifying the expression levels of the CYP19A gene. Studies
269 in different model organisms support the concept that PFOA mimics the activity of

estrogens, leading to the disruption of the hypothalamic-pituitary-gonadal (HPG) endo- crine axis [30,40]. Tilton et al. [13] reported that dietary exposure to PFOA significantly increased expression of the sensitive estrogen biomarker protein vitellogenin (Vtg) in rainbow trout, and induced an overall hepatic gene expression profile highly similar to that of 17b-estradiol. Liu et al. [11] stated that PFOA increased Vtg expression in cultured tilapia hepatocytes, and Wei et al. [12] showed that PFOA increased Vtg expression and caused disruption of gonad development in rare minnows (*Gobiocypris rarus*). Although Vtg expression was not investigated in the present study, our results confirm that PFOA acts as an endocrine disruptor in the gonads of carp by increasing the expression level of the CYP19A gene in testes and reducing it in ovaries. Most importantly, CYP19A expression levels in both male and female gonads did not differ significantly between carp exposed to the environmental dose of 200 ng/L PFOA and those exposed to the experimen- tal dose of 2 mg/L. These data suggest that the environmental dose of 200 ng/L of PFOA is nearly as effective as the experimental dose of 2 mg/L in causing endocrine disruption in the male and female gonads of carp. We may speculate that PFOA functions as physiologically active hormones that have biological effects at low doses. In this context, a few molecules of PFOA may be sufficient to saturate target receptors and elicit the downstream response.

Overall, the present study shows that PFOA at the environmental dose of 200 ng/L does not accumulate in carp tissues; nevertheless, it produces the same changes in gene expression as those at the experimental dose of 2 mg/L. Notably, whereas environmental doses of PFOA do not seem to affect the detoxifying activity of the liver (i.e., GST is efficiently expressed), they act as an endocrine disruptor in male and female gonads by dysregulating the expression levels of CYP19A. Given that many recent studies in animal models, as well as in human populations, have highlighted the adverse effects of PFOA in relation to its bioaccumulation, our data emphasize that environmental PFOA levels dysregulate the HPG axis, in the absence of measurable bioaccumulation. Therefore, in the case of the carp and fish in general that live in aquatic environments containing PFOA, the risk exists of permanently altering the HPG axis and leading ultimately to effects on the reproductive system.

Whether altered CYP19A expression in male and female carp exposed to the environmental dose of 200 ng/L of PFOA can also affect fish reproductive potential (fecundity, hatching, growth, or development/sexual maturation) was not evaluated in the present study. It is known, however, that treatment with experimental doses of PFOA alters the sexual organs in male and female rare minnows, in the way that oocytes develop in testis and ovaries undergo degeneration [12]. Moreover, PFOA parental exposure of Japanese medaka resulted in adverse effects to offspring, leading to increased mortality in the F1 fish generation [41]. On the other hand, Hagenaars et al. reported no effects on zebrafish reproduction after exposure at 0.1-, 0.5-, and 1-mg/L doses for 1 mo [39]. Although fish reproductive endocrinology has some unique aspects, the basic structure and function of the HPG axis is well conserved across all vertebrates. Hence the results obtained with endocrine disruptor compounds in fish models might be extrapolatable to other species. Furthermore, because humans may be exposed to PFOA (although through pathways different from in fish), the causal relationships presented in the present study might be analogous to those described in humans [42]. Notably, in recent years, increases in endocrine disorders such as precocious puberty, menopause, and infertility have been suspected of being caused by exposure to endocrine disruptors [43–46]. Moreover, it is well known that endocrine disruptors that mimic natural hormones exert their biological effects at similarly low doses. Therefore, PFOA's estrogenic activity combined with its capability to induce biological effects in the range of environmental exposures should be seriously considered as a possible factor in the development of human diseases.

In conclusion, the present study reports that environmental concentrations of PFOA do not accumulate in tissues after 56 d of exposure but PFOA does act as an endocrine disruptor in male and female carp. Such evidence raises concerns that exposure to environmental doses of PFOA may affect gene expression in other animals and humans. Further studies are required to identify additional exposure pathways and determine if similar changes in gene expression occur in other animals and humans.

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328 **References**

329 [1] Suja F, Pramanik BK, Zain SM. 2009. Contamination, bioaccumulation and toxic
330 effects of perfluorinated chemicals (PFCs) in the water environment: A review paper.
331 Water Sci Technol 60:1533–1544.
332 [2] Lindstrom AB, Strynar MJ, Libelo EL. 2011. Polyfluorinated compounds: Past,
333 present, and future. Environ Sci Technol 45:7954–7961.
334 [3] Post GB, Cohn PD, Cooper KR. 2012. Perfluorooctanoic acid (PFOA), an emerging
335 drinking water contaminant: A critical review of recent literature. Environ Res 116:93–
336 117.
337 [4] Eschauzier C, Raat KJ, Stuyfzand PJ, De Voogt P. 2013. Perfluorinated alkylated
338 acids in groundwater and drinking water: Identification, origin and mobility. Sci Total
339 Environ 458-460:477–485.
340 [5] Kannan K, Newsted J, Halbrook RS, Giesy JP. 2002. Perfluorooctane- sulfonate and
341 related fluorinated hydrocarbons in mink and river otters from the United States. Environ
342 Sci Technol 36:2566–2571.
343 [6] Giari L, Guerranti C, Perra G, Lanzoni M, Fano EA, Castaldelli G. 2015.
344 Occurrence of perfluorooctanesulfonate and perfluorooctanoic acid and histopathology
345 in eels from north Italian waters. Chemosphere 118:117–123.
346 [7] Olsen GW, Huang HY, Helzlsouer KJ, Hansen KJ, Butenhoff JL, Mandel JH. 2005.
347 Historical comparison of perfluorooctanesulfonate, perfluor- ooctanoate, and other
348 fluorochemicals in human blood. Environ Health Perspect 113:539–545.
349 [8] Ankley GT, Kuehl DW, Kahl MD, Jensen KM, Linnum A, Leino RL, Villeneuvet
350 DA. 2005. Reproductive and developmental toxicity and bioconcentration of
351 perfluorooctanesulfonate in a partial life-cycle test with the fathead minnow (Pimephales
352 promelas). Environ Toxicol Chem 24:2316–2324.

353 [9] Guruge KS, Yeung LW, Yamanaka N, Miyazaki S, Lam PK, Giesy JP, Jones PD,
354 Yamashita N. 2006. Gene expression profiles in rat liver treated with perfluorooctanoic
355 acid (PFOA). Toxicol Sci 89:93–107.
356 [10] Lau C, Anitole K, Hodes C, Lai D, Pfahles-Hutchens A, Seed J. 2007. Perfluoroalkyl
357 acids: A review of monitoring and toxicological findings. Toxicol Sci 99:366–394.
358 [11] Liu C, Yu K, Shi X, Wang J, Lam PK, Wu RS, Zhou B. 2007. Induction of oxidative
359 stress and apoptosis by PFOS and PFOA in primary cultured hepatocytes of freshwater
360 tilapia (Oreochromis niloticus). Aquat Toxicol 82:135–143.
361 [12] Wei Y, Dai J, Liu M, Wang J, Xu M, Zha J, Wang Z. 2007. Estrogen-like properties
362 of perfluorooctanoic acid as revealed by expressing hepatic estrogen-responsive genes in
363 rare minnows (Gobiocypris rarus). Environ Toxicol Chem 26:2440–2447.
364 [13] Tilton SC, Orner GA, Benninghoff AD, Carpenter HM, Hendricks JD, Pereira CB,
365 Williams DE. 2008. Genomic profiling reveals an alternate mechanism for hepatic tumor
366 promotion by perfluorooctanoic acid in rainbow trout. Environ Health Perspect
367 116:1047–1055.
368 [14] Du Y, Shi X, Liu C, Yu K, Zhou B. 2009. Chronic effects of water-borne PFOS
369 exposure on growth, survival and hepatotoxicity in zebrafish: A partial life-cycle test.
370 Chemosphere 74:723–729.
371 [15] Quinete N, Wu Q, Zhang T, Yun SH, Moreira I, Kannan K. 2009. Specific profiles
372 of perfluorinated compounds in surface and drinking waters and accumulation in mussels,
373 fish, and dolphins from southeastern Brazil. Chemosphere 77:863–869.
374 [16] Han J, Fang Z. 2010. Estrogenic effects, reproductive impairment and developmental
375 toxicity in ovoviparous swordtail fish (Xiphophorus helleri) exposed to perfluorooctane
376 sulfonate (PFOS). Aquat Toxicol 99:281–290.

377 [17] Inoue Y, Hashizume N, Yakata N, Murakami H, Suzuki Y, Kikushima E, Otsuka M.
378 2012. Unique physicochemical properties of perfluorinated compounds and their
379 bioconcentration in common carp Cyprinus carpio L. Arch Environ Contam Toxicol
380 62:672–680.
381 [18] Yang B, Zou W, Hu Z, Liu F, Zhou L, Yang S, Kuang H, Wu L, Wei J, Wang J, Zou
382 T, Zhang D. 2014. Involvement of oxidative stress and inflammation in liver injury caused
383 by perfluorooctanoic acid exposure in mice. Biomed Res Int 2014:409837.
384 [19] Mortensen ASl, Letcher RJ, Cangialosi MV, Chu S, Arukwe A. 2011.
385 Tissue bioaccumulation patterns, xenobiotic biotransformation and steroid hormone
386 levels in Atlantic salmon (Salmo salar) fed a diet containing perfluoroactane sulfonic or
387 perfluorooctane carboxylic acids. Chemosphere 83:1035–1044.
388 [20] Rosenmai AK, Nielsen FK, Pedersen M, Hadrup N, Trier X, Christensen JH,
389 Vinggaard AM. 2013. Fluorochemicals used in food packaging inhibit male sex hormone
390 synthesis. Toxicol Appl Pharm 266:132–142.
391 [21] Yang B, Wang X, Jiang J, Cheng X. 2013. Involvement of CD244 in regulating
392 CD4þ T cell immunity in patients with active tuberculosis. PLoS One 8:e63261.
393 [22] Loos R, Locoro G, Huber T, Wollgast J, Christoph EH, de Jager A, Manfred Gawlik
394 B, Hanke G, Umlauf G, Zald'var JM. 2008. Analysis of
395 perfluorooctanoate (PFOA) and other perfluorinated compounds
396 (PFCs) in the River Po watershed in N-Italy. Chemosphere 71:306–313.
397 [23] Loos R, Gawlik BM, Locoro G, Rimaviciute E, Contini S, Bidoglio G.
398 2009. EU-wide survey of polar organic persistent pollutants in European river waters.
399 Environ Pollut 157:561–568.
400 [24] Oakes KD, Sibley PK, Martin JW, Solomon KR, Mabury SA, Van der Kraak GJ.
401 2004. Impact of perfluorooctanoic acid on fathead minnow (Pimephales promelas) fatty

acyl-CoA oxidase activity, circulating steroids, and reproduction in outdoor microcosms. Environ Toxicol Chem 23:1912–1919.

[25] Kim WK, Lee S-K., Jung J. 2010. Integrated assessment of biomarker responses in common carp (Cyprinus carpio) exposed to perfluorinated organic compounds. J Hazard Mater 180:395–400.

[26] Guerranti C, Palmieri M, Mariottini M, Focardi SE. 2011. Persistent organic pollutants in human milk from central Italy: Levels and time trends. ISRN Toxicol 2011:107514.

[27] Guerranti C, Ancora S, Bianchi N, Perra G, Fanello EL, Corsolini S, Fossi MC, Focardi SE. 2013. Perfluorinated compounds in blood of Caretta caretta from the Mediterranean Sea. Mar Pollut Bull 73:98–101.

[28] Guerranti C, Perra G, Corsolini S, Focardi SE. 2013. Pilot study on levels of perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) in selected foodstuffs and human milk from Italy. Food Chem 140:197–203.

[29] Rotondo JC, Borghi A, Selvatici R, Magri E, Bianchini E, Montinari E, Corazza M, Virgili A, Tognon M, Martini F. 2016. Hypermethylation- induced inactivation of the IRF6 gene. JAMA Dermatol 152:1–5.

[30] Benninghoff AD, Orner GA, Buchner CH, Hendricks JD, Duffy AM, Williams DE. 2012. Promotion of hepatocarcinogenesis by perfluor- oalkyl acids in rainbow trout. Toxicol Sci 125:69–78.

[31] Das S, Mukherjee D. 2013. Effect of cadmium chloride on secretion of 17beta-estradiol by the ovarian follicles of common carp. Cyprinus carpio. Gen Comp Endocrinol 181:107–114.

[32] Amado LL, Garcia ML, Pereira TC, Yunes JS, Bogo MR, Monserrat JM.

427 2011. Chemoprotection of lipoic acid against microcystin-induced toxicosis in common
428 carp (Cyprinus carpio, Cyprinidae). Comp Biochem Phys C 154:146–153.
429 [33] Rotondo JC, Bosi S, Bassi C, Ferracin M, Lanza G, Gafa' R, Magri E, Selvatici R,
430 Torresani S, Marci R, Garutti P, Negrini M, Tognon M, Martini F. 2015. Gene expression
431 changes in progression of cervical neoplasia revealed by microarray analysis of cervical
432 neoplastic keratinocytes. J Cell Physiol 230:806–812.
433 [34] Nakayama S, Strynar MJ, Helfant L, Egeghy P, Xibiao Y, Lindstrom AB. 2007.
434 Perfluorinated compounds in the Cape Fear drainage basin in North Carolina. Environ Sci
435 Technol 41:5271–5276.
436 [35] Organisation for Economic Co-operation and Development. 2006. SIDS initial
437 assessment report after SIAM 22—Ammon Perfluor & Perfluor Acid. Paris, France.
438 [36] Kurume Laboratory. 2001. Bioaccumulation test of perfluoroalkylcar- boxylic acid
439 (C = 7–13) (This test was performed using perfluorooctanoic acid [test substance number
440 K-1519].) in carp. Chemicals Evaluation and Research Institute, Tokyo, Japan.
441 [37] Inoue Y, Hashizume N, Yakata N, Murakami H, Suzuki Y, Kikushima E, Otsuka M.
442 2012. Unique physicochemical properties of perfluorinated compounds and their
443 bioconcentration in common carp Cyprinus carpio L. Arch Environ Contam Toxicol
444 62:672–680.
445 [38] Mate's JM. 2000. Effects of antioxidant enzymes in the molecular control of
446 reactive oxygen species toxicology. Toxicology 153:83–104.
447 [39] Hagenaars A, Vergauwen L, Benoot D, Laukens K, Knapen D. 2013. Mechanistic
448 toxicity study of perfluorooctanoic acid in zebrafish suggests mitochondrial dysfunction to
449 play a key role in PFOA toxicity. Chemosphere 91:844–856.
450 [40] Lee JW, Lee JW, Shin YJ, Kim JE, Ryu TK, Ryu J, Lee J, Kim P, Choi K, Park K.
451 2017. Multi-generational xenoestrogenic effects of perfluor- oalkyl acids (PFAAs)

452 mixture on Oryzias latipes using a flow-through exposure system. Chemosphere 169:212–
453 223.
454 [41] Ji K, Kim Y, Oh S, Ahn B, Jo H, Choi K. 2008. Toxicity of perfluorooctane sulfonic
455 acid and perfluorooctanoic acid on freshwater macroinverte- brates (Daphnia magna and
456 Moina macrocopa) and fish (Oryzias latipes). Environ Toxicol Chem 27:2159–2168.
457 [42] White SS, Fenton SE, Hines EP. 2011. Endocrine disrupting properties of
458 perfluorooctanoic acid. J Steroid Biochem Mol Biol 127:16–26.
459 [43] Gore AC, Walker DM, Zama AM, Armenti AE, Uzumcu M. 2011. Early life
460 exposure to endocrine-disrupting chemicals causes lifelong molecular reprogramming of
461 the hypothalamus and premature reproductive aging. Mol Endocrinol 25:2157–2168.
462 [44] Deng F, Tao FB, Liu DY, Xu YY, Hao JH, Sun Y, Su PY. 2012. Effects of growth
463 environments and two environmental endocrine disruptors on children with idiopathic
464 precocious puberty. Eur J Endocrinol 166:803–809.
465 [45] Fowler PA, Bellingham M, Sinclair KD, Evans NP, Pocar P, Fischer B, Schaedlich
466 K, Schmidt JS, Amezaga MR, Bhattacharya S, Rhind SM, O'Shaughnessy PJ. 2012.
467 Impact of endocrine-disrupting compounds (EDCs) on female reproductive health. Mol
468 Cell Endocrinol 355:231–239.
469 [46] Marques-Pinto A, Carvalho D. 2013. Human infertility: Are endocrine disruptors to
470 blame? Endocr Connect 2:15–29.
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Table 1. Perfluorooctanoic acid nominal and measured test concentrations in the test tanks[a].

Nominal test concentration	Measured test concentration[b]
2 mg/L	1.932 ± 0.023 mg/L
200 ng/L	182.53 ± 12.16 ng/L
0 ng/L (unexposed)	3.49 ± 0.55 ng/L

[a]Measures were taken at the beginning (day 1), middle (day 28), and end (day 56) of the exposure period.

[b]Values are mean ± standard deviation (SD) for n = 3.

480 Table 2. Perfluorooctanoic acid (PFOA) concentrations[a] in samples of carp exposed to 2 mg l^{-1} for 56
481 d[b].

Tissue/Organ	Range values (ng g^{-1})	Mean ± SE (ng g^{-1}) (all fish n = 10)	Mean ± SE (ng g^{-1}) (females n = 5)	Mean ± SE (ng g^{-1}) (males n = 5)
blood	21.8 - 97.8	64.9 ± 7.3A	66.3 ± 9.3A	63.2 ± 12.7A
liver	21.4 - 38.9	28.1 ± 1.6A	27.6 ± 2.6A	28.8 ± 1.9A
gonad	0.9 - 11.5	3.7 ± 0.9A	3.6 ± 1.6A	3.3 ± 5A

482 [a]ng g^{-1} wet weight

483 [b]All the values were above the limit of detection (0.4 ng g^{-1} wet wt). A indicates significant
484 differences in PFOA concentrations among diverse tissues/organs. No disparities between the sexes in
485 any tissue/organ were found using the Student's t test.

486 *$p < 0.05$.

487 SE = standard error

488

Figure legends

Figure 1. Expression levels of glutathione S-transferase mRNA measured with real-time quantitative polymerase chain reaction in liver tissues from carp. The white bar represents unexposed carp; the gray bar indicates carp exposed to the environmental dose of 200 ng/L of perfluorooctanoic acid (PFOA); and the black bar denotes carp exposed to the experimental dose of 2 mg/L of PFOA. Relative expression was calculated by use of the comparative threshold (DDCt) method. Data are expressed as a relative-fold change (2^{-DDCt}) over the value of unexposed carp. Error bars represent standard error of mean for $n = 10$. The glyceraldehyde 3-phosphate dehydrogenase gene was used as internal control. All reactions were performed in triplicate and results were averaged. $p < 0.0001$ versus carp exposed to the environmental dose of 200 ng/L of PFOA and versus unexposed carp; $^{D}p < 0.05$ versus unexposed carp.

Figure 2. Expression levels of CYP19A mRNA measured with real-time quantitative polymerase chain reaction in testes tissues from male carp. The white bar depicts unexposed carp; the grayish bar identifies carp exposed to the environmental dose of 200 ng/L of perfluorooctanoic acid (PFOA); and the black bar represents carp exposed to the experimental dose of 2 mg/L of PFOA. Error bars are ± standard error for $n = 5$. $p < 0.0001$ versus unexposed carp; $^{D}p < 0.05$ versus unexposed carp.

Figure 3. Expression levels of CYP19A mRNA measured with real-time quantitative polymerase chain reaction in ovarian tissues from female carp. The white bar shows unexposed carp; the grayish bar represents carp exposed to the environmental dose of 200 ng/L of perfluorooctanoic acid (PFOA); and the black bar denotes carp exposed to the experimental dose of 2 mg/L of PFOA. Error bars represent ± standard error for $n = 5$. $p < 0.0001$ versus unexposed carp; $^{D}p < 0.0001$ versus unexposed carp.