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1 ENDOCRINE DISRUPTION IN WILD POPULATIONS OF CHUB (LEUCISCUS
2 CEPHALUS) IN CONTAMINATED FRENCH STREAMS

3

4

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26

27 ABSTRACT

28

29 The aim of this study was to assess endocrine disruptive effects in wild population of fish in
30 five French rivers selected to represent different pollution contexts at two seasons (summer
31 and fall). For that purpose, a panel of biometrical parameters (length, weight, gonado-somatic
32 index: GSI) and biochemical (ethoxyresorufin-O-deethylase: EROD, vitellogenin: VTG, brain
33 aromatase) and histological biomarkers (gonads histology) was used in chub (Leuciscus
34 cephalus), a common cyprinid fish species. In fish from the reference site, EROD activity and
35 VTG levels were low at the two seasons. Brain aromatase activities (AA) were similar to
36 other species and increased with increasing GSI and gonad maturation. Among the four
37 contaminated sites, the Jalle d'Eysines River was the most impacted site. At this site, fish
38 were exposed to estrogenic substances as demonstrated by the VTG induction in males and
39 the arrest of development of the gonads that led to lower brain AA compared to fish from the
40 reference site. In fish from other contaminated sites, EROD activity was induced as compared
41 to fish from the reference site and some males had elevated concentrations of VTG.
42 Moreover, the presence of aromatase inhibiting compounds was demonstrated in the
43 sediments of three contaminated sites, even if the precise nature of contaminants is not
44 known. This study provides new data concerning endocrine disruption in wild fish
45 populations inhabiting French rivers and demonstrates that measurements of in vivo and in
46 vitro aromatase could be used as biomarkers of endocrine disruption in field studies.

47

48 Key-words : endocrine disruption, chub, aromatase, vitellogenin, histology

49

50 Introduction

51

52 It has now been clearly established that a number of chemical compounds and natural
53 substances present in the aquatic environment are able to disturb the normal physiology and
54 endocrinology of organisms (Arukwe and Goksoyr, 1998; Sumpter, 1998). These compounds
55 are known as endocrine disrupting chemicals (EDCs) and are of different origins. Among
56 them are natural and synthetic hormones, plant components, pesticides, compounds used in
57 the plastic industry and in consumer products, as well as industrial by-products (Lavado *et al.*,
58 2004). All these EDCs could enter the aquatic environment via effluents or surface runoff,
59 and thus fish will inevitably be exposed. Several studies have documented reproductive
60 disorders and/or endocrine effects in different species of fish exposed to sewage treatment
61 works (STW) effluents or to pulp and paper mill effluents (Marshall Adams *et al.*, 1992;
62 Munkittrick *et al.*, 1998; Karels *et al.*, 1999; Hecker *et al.*, 2002). However, the cause-effect
63 relationship is difficult to establish due to the complexity of the endocrine system and the
64 diversity of modes of action of the EDCs (Lister and Van Der Kraak, 2001).

65 Despite this complexity, a great number of studies have described endocrine disruption in fish
66 from America, Canada, and Europe (for review see Jobling *et al.*, 1998; Vos *et al.*, 2000;
67 Jobling and Tyler, 2003). Information on the status of French rivers regarding endocrine
68 disruption is quite scarce. Studies showed the presence of estrogenic compounds in effluents
69 (Balaguer *et al.*, 1999) and the contamination of surface water and sediments by endocrine
70 disrupters and more particularly estrogenic substances (Labadie and Budzinski, 2005; Pillon
71 *et al.*, 2005). In fish, estrogenic effects have been shown by induction of plasmatic
72 vitellogenin (VTG) in wild males of stickleback (*Gasterosteus aculeatus*) and chub (*Leuciscus*
73 *cephalus*) sampled in rivers under urban pressure (Flammarion *et al.*, 2000; Sanchez *et al.*,
74 2008). Androgenic effects were also highlighted by the induction of spiggin in river under

75 urban and industrialized pressure (Sanchez *et al.*, 2008). However, these studies are scarce
76 and to our knowledge, other alterations of the endocrine system have not been explored.

77 In this study, we investigated the potential alterations of the endocrine system and of the
78 xenobiotic metabolism in wild fish from different French rivers, one reference and four
79 contaminated sites that reflect different anthropogenic pressures (Table I). Chub (Leuciscus
80 cephalus) was selected as a sentinel species due to its widespread distribution along the study
81 areas and because it is a common non-migratory freshwater cyprinid species that inhabits both
82 clean and polluted rivers (Flammarion and Garric, 1997). A set of biometrical measurements
83 and biochemical biomarkers representing different biological functions were used to assess *in*
84 *situ* impacts of EDCs in fish. Physiological parameters such as length, weight and gonad
85 somatic index (GSI) were determined. Histological analysis of gonads was performed to
86 determine the sex and the maturation state of the fish. The biochemical biomarkers included
87 EROD activity (7-ethoxyresorufin-O-deethylase, a cytochrome P450 1A monooxygenase
88 activity) a biomarker of exposure to AhR (Aryl Hydrocarbons receptor) ligands such as
89 dioxins, polycyclic aromatic hydrocarbons (PAHs) and polychlorobiphenyls (PCBs) (Whyte
90 *et al.*, 2000), and plasmatic VTG in males, as a marker of exposure to xeno-estrogens
91 (Flammarion *et al.*, 2000). Vitellogenin is the yolk protein synthesized by the liver in females
92 that serves as food reserve for the developing embryos. In male fish, very few, if any,
93 vitellogenin can be detected. Although vitellogenin is normally not present in male fish, its
94 synthesis can be easily induced by estrogens in laboratory experiments (Brion *et al.*, 2002)
95 and be used as a biomarker of estrogenic exposure in field studies (Sumpter and Jobling,
96 1995; Flammarion *et al.*, 2000). Additionally, the activities of brain cytochrome P450
97 aromatase, a key steroidogenic enzyme responsible for the irreversible conversion of
98 androgens to estrogens, were also measured in male and female fish. It is now known that
99 EDCs can affect aromatase expression and/or activity through different modes of action

100 (Cheshenko et al., 2008, Hinfrey et al., 2008). The positive estrogenic regulation of brain
101 aromatase cyp19a1b gene expression in different fish species is well demonstrated (Callard *et*
102 *al.*, 2001; Menuet *et al.*, 2005; Hinfrey *et al.*, 2006b), this up-regulation requiring binding of
103 liganded-ER on ERE located in the promoter region of the gene (Menuet et al., 2005). On the
104 contrary, several classes of compounds such as dioxin-like compounds, organotins or
105 imidazole-like fungicides have been shown to interfere negatively either with the aromatase
106 gene expression or the aromatase enzymatic complex to inhibit aromatase activity (Monod *et*
107 *al.*, 1993; Sanderson *et al.*, 2002; Hinfrey *et al.*, 2006a; Laville *et al.*, 2006, Cheshenko *et al.*,
108 2007). Perturbations of aromatase activities have also been reported in wild fish populations
109 living in contaminated areas (Noaksson *et al.*, 2001; Orlando *et al.*, 2002; Lavado *et al.*, 2004;
110 Hecker *et al.*, 2007). Additionally, the aromatase-inhibiting potency of sediments extracts
111 collected at the study sites was assessed in vitro in a rainbow trout in vitro microsomal
112 aromatase activity assay previously developed in our laboratory (Hinfrey *et al.*, 2006a). To
113 achieve this goal, we first developed the VTG and the aromatase activity assays for chub and
114 checked their responsiveness and sensitivity after exposure to a model compound,
115 ethinylestradiol. Our aims were (i) to acquire data on endocrine disruption in male and female
116 fish from reference and contaminated French rivers, (ii) to assess pathways of endocrine
117 disruption other than VTG synthesis perturbations to extend fish exposure assessment to non
118 estrogenic substances, (iii) to determine the extent to which aromatase could be a sensitive
119 biomarker for field studies.

120

121 Materials and methods

122

123 **Laboratory exposure to EE2**

124 To validate chub VTG and aromatase activity assays, we exposed fish to a model endocrine
125 disrupting compound, ethynilestradiol (EE2), as a typical inducer of VTG synthesis and brain
126 aromatase activities.

127 24 adult wild chubs were collected in September 2007 from two different moderately
128 contaminated sites under urban and agricultural pressure. Fish were maintained in the
129 laboratory for 2 months in clear water for depuration.

130 Two groups of 12 fish were randomly held in two glass aquaria filled with 200 L of water.
131 They were fed a commercial pellet diet everyday (about 1% total body weight). After an
132 acclimation period of 4 days, they were exposed to EE2 (nominal concentration of 50 ng/L
133 in DMSO) or DMSO alone (0.01% v/v) in water for 21 days. The exposure was carried out in
134 semi-static conditions and 150 L of water was renewed every day. Photoperiod was 16 h
135 night-8 h day. Physicochemical parameters were measured each day (temperature 16.9 ± 0.6
136 °C; pH 8.0 ± 0.2 ; dissolved oxygen 7.7 ± 0.4 mg/L; conductivity 316 ± 34 μ S/cm [mean \pm
137 SD]).

138

139 **Sampling of wild fish**

140 Male and female wild chub were collected by electrofishing in five different French rivers in
141 summer (May/June) and fall (September/October) 2006. According to Guerriero *et al.* (2005),
142 for common chub these periods correspond to spawning and post-spawning periods
143 respectively. The sampling stations were characterized by different environmental influences
144 (industrialized, urban, agricultural pressures) and are described in the Table 1.

145

146 **Tissues sampling**

147 Dissection was carried out immediately after the fish were caught. A blood sample was taken
148 directly from the caudal vein using a heparinised syringe. Blood samples were centrifuged for
149 10 min (3000g, 4°C) and the plasma were stored at -80°C until VTG analysis. Total body
150 length (cm), body weight (g) and macroscopic sex were recorded for each individual. The
151 gonado-somatic index (GSI = gonad weight expressed as a percentage of total body weight)
152 was calculated for each fish. Small pieces of liver were removed, placed in 2 ml tubes with
153 glass grinding balls containing phosphate buffer (pH 7.6) and snapped frozen in liquid
154 nitrogen before storage at -80°C until EROD activity analysis. Gonads were removed and
155 fixed in formaldehyde (4%) until histological analysis. Brains were sampled and
156 homogenized with a Teflon potter homogenizer in a 50 mM potassium phosphate buffer, pH
157 7.4, containing 1 mM PMSF, 1 mM EDTA and 20% glycerol (v/v) in a ratio of 1:2 (w/v). The
158 homogenates were then frozen in liquid nitrogen and stored at -80°C until aromatase activity
159 (AA) analysis.

160

161 **EROD activity assay**

162 Frozen livers were homogenized for 2 x 10 seconds with an automated grinding ball
163 homogeniser (Precellys® 24, Bertin Technologies, France). The homogenates were
164 centrifuged (10,000 g, 15 min, 4 °C) to obtain the post-mitochondrial (S9) fraction. Total
165 protein concentrations of these fractions were determined using bovine serum albumin
166 (Sigma-Aldrich, France) as a standard (Bradford, 1976). EROD assay was adapted from
167 (Flammarion *et al.*, 1998) with minor modifications for substrate concentrations. Briefly, 10
168 µl of diluted liver S9 fraction were added to 200 µl of 7-ethoxyresorufin (8 µM final
169 concentration) and 10 µl of β-NADPH (0.5 mM final concentration) to start the reaction.

170 Fluorescence kinetics were measured in a black microplate with a spectrofluorimeter (Safire²,
171 Tecan, Austria).

172

173 **Chub VTG assay**

174 A homologous competitive ELISA assay was specifically developed to quantify VTG in chub
175 plasma. For that purpose, VTG was purified from E2-induced chubs according to Brion *et al.*
176 (2000) and polyclonal anti-chubVTG antibodies were raised in rabbits as previously described
177 (Brion *et al.*, 2002). Specificity of the anti-chubVTG antibodies was verified and an
178 homologous chub VTG competitive ELISA assay was developed and validated according to
179 Brion *et al.* (2002). Briefly, Nunc Maxisorp microtiter plates (Nunc Roskilde, Denmark) were
180 coated with 100 µL of Chub-Vtg at 100 µg/ml in 0.05 M carbonate-bicarbonate (pH 9.6).
181 Standard of Vtg was serially half-diluted from 0.98 to 1000 ng/ml, and plasma samples were
182 serially third-diluted five times from an initial dilution of 1:7.5 for males and 1:25 for female.
183 Standards and plasma samples were preincubated with the primary antibody (final dilution of
184 1:80000 in PBS, 1% BSA). Pre-coated ELISA microplates were incubated with preincubated
185 primary antibody solution with standards or samples (100 µl) and then with secondary
186 antibody (horseradish peroxidase goat anti-rabbit IgG diluted 1:2000 in PBS, 1% BSA). The
187 peroxidase activity was revealed by adding 100 µl of tetramethylbenzidine enzyme substrate
188 (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) and was stopped by addition of
189 50 µl of 1 M phosphoric acid (H₃PO₄). The absorbance was read at 450 nm. Calculation was
190 performed as described by Brion *et al.* (2002).

191

192 **Brain aromatase activity assay**

193 Brain homogenates were centrifuged (10,000 g, 20 min, 4 °C) to obtain S9 fractions. Total
194 protein concentrations of the S9 fractions were determined using bovine serum albumin
195 (Sigma-Aldrich, France) as a standard (Bradford, 1976).

196 The AA was measured as the specific release of tritiated water accompanying the conversion
197 of [1 β -³H(N)]androstenedione to estrone as described in Hinfray *et al.* (2006a) with some
198 modifications. Indeed, optimal conditions to measure chub brain AA were first determined,
199 including duration and temperature of incubation, concentration of S9 protein, and
200 concentration of labeled substrate.

201 Briefly, 500 μ g of brain S9 protein were added to a potassium phosphate buffer (50mM)
202 containing 1 mM of NADPH. The reaction was started by adding of 150 nM of [1 β -
203 ³H(N)]androst-4-ene-3,17-dione. After 1 h at 18°C the reaction was stopped and the aqueous
204 fraction was extracted twice with chloroform and once with charcoal (5% w/v) to remove any
205 remaining organic compounds. Then, the radioactivity of the aqueous fraction was read in a
206 Liquid Scintillation Counter (Microbeta, Perkin Elmer).

207 The aromatase assay was demonstrated to be linear with time and the amount of protein.
208 Appropriate background and control incubations were routinely performed.

209

210 **Gonad histology**

211 After fixing the gonads in formaldehyde 4%, three pieces of the gonad were dehydrated
212 through a series of graded ethanol, cleared with toluene and embedded in paraffin. Sections
213 were 7 μ m thick, stained with haematoxylin-eosin-saffron, and observed under an optical
214 microscope.

215 Ovarian maturation was divided into three stages: primary oocytes (only oogonia and primary
216 oocytes), early vitellogenic stage (primary oocytes, and secondary oocytes), and advanced
217 vitellogenic stage (a great majority of secondary oocytes). Testicular maturation was also

218 divided into three stages according to the predominant cell type in the testis: spermatogonies,
219 spermatocytes and spermatozooids stages. None of the fish testes studied presented a majority
220 of spermatids.

221

222 **In vitro analysis of sediments extracts**

223 Sediments were sampled in the upper 10 cm surface layer (about 500 g), sieved using a 2 mm
224 sieve and stored at -20°C until processing. Extraction of 5 grams of dry sediment was
225 performed by Accelerated Solvent Extraction (ASE) according to Louiz *et al.* (2008). The
226 solvent used for ASE was a heptane:acetone (1:1) mixture. The final extract was dissolved in
227 methanol.

228 The presence of aromatase inhibiting compounds in sediments extracts was assessed by using
229 an in vitro aromatase assay previously developed (Hinfray *et al.*, 2006a). Sediment extracts
230 (0.25 %) were pre-incubated with rainbow trout (rt) brain microsomes during 1 h at 27°C in a
231 potassium phosphate buffer (50mM) containing 1 mM NADPH. The aromatization reaction
232 was then started by the addition of 75 nM of [1 β -³H(N)]androst-4-ene-3,17-dione. After 30
233 min, the reaction was stopped. The aqueous fraction was extracted and the radioactivity was
234 read as described above for chub brain AA. Three independent experiments were performed
235 for each sediment extract.

236

237 **Statistical analysis**

238 Statistical analyses were performed with SPSS software (SPSS Inc, Chicago IL, USA). Data
239 of EROD activity, VTG synthesis and in vivo AA were subjected to a logarithmic
240 transformation to stabilize the variance and obtain a normal distribution. We verified that
241 log(1+data) followed a normal distribution (Khi2 test for normality, p < 0.05) and that
242 variance was homogeneous (Levene's test, p < 0.05). Data sets exhibiting normal distribution

243 after log transformation were subjected to a one-way analysis of variance (ANOVA) using
244 sites as factor followed by a Tuckey's test ($p < 0.05$). Data sets with no normal distribution
245 were subjected to a non parametric test of Kruskal-Wallis followed by a Mann-Whitney test
246 ($p < 0.05$). Males and females were treated separately.
247 For gonad stage of development analysis, differences of proportions between the reference
248 site and the contaminated sites were elucidated by the Fisher's test ($p < 0.05$).
249 The effects of sediment extracts on in vitro AA were expressed as a percentage of the solvent
250 control AA. Due to small number of assays ($N = 3$), statistical analysis was performed using
251 the Student's t test ($p < 0.05$).

252

253 **Results**

254

255 **Laboratory exposure to EE2**

256

257 Analysis of biometric parameters of male chub exposed to 50 ng/L EE2 for 21 days revealed
258 no difference compared to the control males, while exposed females had lower length, weight
259 and GSI values when compared to the control females (Table 2). In control chub, VTG
260 concentrations ranged between 500 and 8400 ng/ml for males and between 0.6 and 25 µg/ml
261 for females. Exposure to EE2 induced VTG synthesis around 13000 and 7500 fold in males
262 and females, respectively (Table 3). Concentrations of VTG in EE2 exposed fish get from
263 23243 to 36128 µg/ml. Brain AA were also induced by exposure to EE2. Brain AA were
264 about 4-fold and 2.6-fold higher in exposed males and females chub respectively compared to
265 control chubs (Table 3). EROD activities were strongly inhibited in both male and female
266 chubs (Table 3). Overall, these results showed the suitability of the biochemical assays
267 established for chub and the responsiveness of this fish species to a model EDC.

268

269 **Field study**

270

271 Morphometrical parameters

272 The main morphometric characteristics of chub collected in the different study sites are given
273 in Table 4. Male and female fish from the Drôme River (reference site) had lower GSI values
274 in fall than in summer. All the fish from the contaminated sites exhibited similar values of
275 GSI in summer and fall, except female fish from the Nonette River that exhibited higher GSI
276 values in fall.

277 Males collected in the summer were homogeneous in size and weight; however, fish from all
278 contaminated sites presented lower GSI values as compared to fish from the reference site
279 (Drôme). During the fall, males caught in all contaminated sites were longer and heavier than
280 those from the Drôme River, but only males from the Nonette River had higher GSI values.
281 For females collected in the summer, only those from the Rhône and Jalle d'Eysines rivers
282 were different from the reference site (Drôme). They had higher size and weight but lower
283 GSI values. In the fall, females from the Rhône and the Nonette rivers were longer and/or
284 heavier than females from the Drôme River. Additionally, females from the Jalle d'Eysines
285 River showed lower GSI values and females from the Nonette river showed higher GSI values
286 compared to females from the reference site.

287

288 Histological analysis

289 Females caught in the reference site were mainly at the advanced vitellogenesis stage in the
290 summer (Figure 1A). In the fall, females were at the primary oocyte stage (33 %) and early
291 vitellogenic stages (67 %) (Figure 1B). Males sampled in the summer in the reference site
292 were all mature (spermatozooids stage) (Figure 1C). In the fall, all males had gonads at the
293 spermatogonies or spermatocytes stages (Figure 1D). In the reference site, fish were clearly at
294 different stages of gonad development between summer and fall.

295 In the summer, all contaminated sites, except the Jalle river, had a majority of females in
296 vitellogenesis (early or advanced vitellogenesis) (Figure 1A). On the contrary, females from
297 the Jalle d'Eysines River were predominantly at the primary oocyte stage. In the fall, females
298 were essentially at the primary oocytes stage in the Jalle d'Eysines River while females from
299 the three other contaminated sites had a high proportion of advanced vitellogenic stages
300 (Figure 1B). The arrest of gonad development in fish from the Jalle d'Eysines River in the
301 summer and the fall is in agreement with the lower GSI observed in these fish.

302 In the contaminated sites, males displayed high proportions of testis at the spermatogonies
303 and spermatocytes stages in summer (Figure 1C). In the fall, they presented testis at the
304 spermatogonies or spermatocytes stages (Figure 1D).

305 Fish from all sites were also examined for the presence of intersex characteristics. Intersex
306 fish were found in only two sites: the Drôme (reference site) and the Rhône rivers. In the
307 summer in the Drôme River, 13% (2/15) of males were intersex with one male with more than
308 10 primary oocytes per slide and one male with only one primary oocyte in one slide. No
309 intersex fish were found in the Drôme River during fall. In the Rhône River, 9% (1/11) and
310 7% (1/15) of males were intersex in the summer and the fall respectively. All of them
311 presented more than 10 primary oocytes per slide. In the three other sites, no intersex fish
312 were found during either season.

313

314 EROD activity

315 Mean EROD activities measured in male and female chubs from the Drôme River were
316 comprised between 0.2 and 2.1 pmol/mg/min (Figure 2A and B). These values are lower but
317 comparable to those measured in Chub from other French unpolluted rivers (2-9
318 pmol/mg/min) (Flammarion and Garric, 1997). In summer, EROD activity was induced in
319 male and female from the Jalle d'Eysine and the Nonette rivers and in females from the Lez
320 river compared to fish from the reference site (Drôme), suggesting an exposure of fish to AhR
321 agonists (Figure 2A and B). The induction factors were comprised between 4 (males from the
322 Jalle d'Eysine river) and 18.7 (females from the Jalle d'Eysine River). In fall, only males
323 from the Nonette River exhibited higher EROD activity than fish from the Drôme River
324 (Figure 2A). No difference was noted for females during the fall. It is interesting to note that
325 EROD activity in females exhibited high inter-individual variations, probably due to the an

326 influence of GSI on EROD activity in females as previously noted by Flammarion *et al.*
327 (1998).

328

329 Plasmatic VTG

330 In the reference site (Drôme river), males exhibited plasmatic VTG concentrations from non
331 detectable levels to 382 ng/ml (Figure 3). Geometric mean plasmatic VTG concentrations
332 were of 93 ng/ml and 76 ng/ml in the summer and the fall respectively. VTG concentrations
333 measured in our study are of the same order as those measured in male cyprinid fish from
334 reference sites such as roach (mean concentrations between 47 and 1502 ng/ml) (Tyler *et al.*,
335 2005) but slightly lower than those measured in chub (mean concentration of 970 ng/ml)
336 (Randak *et al.*, 2009). Only the males from the Jalle d'Eysines River presented statistically
337 significant induced mean VTG concentrations in summer as compared to males from the
338 reference site, with levels of VTG comprise from 82 ng/ml to 8663 ng/ml. In all contaminated
339 sites, mean VTG levels were not statistically different, but some males showed elevated
340 concentrations of VTG compared to males from the reference site that can reach 49 µg/ml,
341 corresponding to level of VTG found in females in control laboratory experiment (Figure 3).
342 Nevertheless, these induced VTG concentrations measured from field chub are lower than
343 those measured in males exposed to 50 ng/L EE2 during 21 days (Table 3).

344

345 Brain aromatase activity

346 In the reference site, high AA was measured in the brain of male and female fish without any
347 difference between sexes in the two seasons. This brain AA varied as maturity of the gonads
348 progresses (Figure 4). In females, brain AA was higher during vitellogenesis as compared to
349 the primary oocytes stage. Despite the variations of AA in females according to the stage of
350 development of the gonads, no difference between seasons was observed in female brain AA

351 (Figure 5). In males from the reference site, brain AA was higher in fish with testis full of
352 spermatozoids (only in summer) as compared to testis at the spermatogony/spermatocyte
353 stages (only in fall) (Figure 4). As a result, a significant effect of the season was noted on
354 brain AA in males with higher AA in the summer than in the fall (Figure 5). At the
355 contaminated sites, brain AA was inhibited in males from the Rhône and Jalle d'Eysines
356 rivers in the summer as compared to brain AA of males from the Drôme river (Figure 5).
357 These brain AA inhibitions led to the suppression (Rhône river) or the inversion (Jalle
358 d'Eysines river) of the seasonal effect on brain AA in the fish from these two sites. No
359 significant effect of site and season was found in female brain AA.

360

361 Effect of sediments extracts on in vitro aromatase activity

362 Sediment extracts of three sites (Rhône, Jalle d'Eysines and Nonette) at the two seasons
363 significantly inhibited in vitro brain AA (between 42% and 76% of inhibition) indicating the
364 presence of aromatase inhibitors in sediments (Figure 6). No effect was detected at the Lez
365 and Drôme sites.

366

367

368 Discussion

369

370 **Methodological developments**

371 In this study, a specific homologous competitive chub-VTG ELISA assay and an aromatase
372 activity assay were first developed and used to assess the effect of a low concentration of
373 ethinylestradiol (EE2). As expected, exposure to 50 ng/L EE2 led to a tremendous VTG
374 synthesis in chub (about 10^4 induction factor). Cyprinid fish such as chub, roach or zebrafish
375 have previously been shown to be sensitive to estrogens exposure (Flammarion *et al.*, 2000;
376 Brion *et al.*, 2004; Lange *et al.*, 2008; Zlabek *et al.*, 2009). In our study, VTG concentrations
377 in exposed males and females reached more than 1 mg/ml supporting similar results observed
378 for chub (Flammarion *et al.*, 2000). It is also interesting to note that the plasmatic VTG
379 concentrations measured in control males from the EE2 exposure experiment (2.1 $\mu\text{g/ml}$)
380 were clearly higher than those of males from reference sites (Drôme river in our study: 93.2
381 ng/ml in summer and 76.1 ng/ml in fall; reference site in the study of Randak *et al.* (2009):
382 970 ng/ml), indicating that even after two months depuration in clean water, VTG
383 concentrations of chubs used as control for the EE2 exposure were not returned to levels
384 found in fish from non contaminated sites. Moreover, brain AA was also induced after
385 exposure to EE2. It is now well demonstrated that brain aromatase cyp19a1b is an estrogen-
386 regulated gene in different fish species (Callard *et al.*, 2001; Menuet *et al.*, 2005; Hinfray *et*
387 *al.*, 2006b), its up-regulation requiring the binding of liganded-ER with ERE and $\frac{1}{2}$ ERE
388 located in the promoter region of the gene (Menuet *et al.*, 2005). Although, to date the chub
389 cyp19a1 genes were not sequenced, our results strongly suggest that chub possess a cyp19a1b
390 gene which is positively regulated by (xeno)-estrogens.

391

392 **Field study**

393 In this study, potential alterations of the endocrine system of wild chub were investigated by
394 using a multi-parametric approach including morphological parameters (length, weight, GSI,
395 gonad histology) and biochemical biomarkers (EROD, VTG, AA). We first looked at all these
396 parameters in fish from the Drôme River to confirm the reference status of this site.

397 **The reference site**

398 The Drôme River in Saillans was chosen as the reference site since it was located in a
399 relatively clean area. Previous studies on PCB and heavy metals concentrations in fish and in
400 sediments showed that the Drôme river is one of the least polluted French river (Flammarion
401 and Garric, 1997; Mazet *et al.*, 2005).

402 As expected, GSI of male and female chub were higher in the summer than in the fall. The
403 GSI well reflected the histological state of the gonads. Female chubs were in full
404 vitellogenesis and male chubs were full of spermatozooids in the summer which correspond to
405 the reproductive period for this species (Guerriero *et al.*, 2005). In the fall, the post spawning
406 period for chub, male and female gonads were at early stages of development. Thus, chub
407 from the Drôme River showed a normal gonadal development. Nevertheless, among male
408 chubs from the Drôme River, the incidence of intersex was 13 %. Intersex fish prevalence
409 observed in wild cyprinid fish population sampled in reference sites are comprised between 0
410 and 41 % for chub, roach and gudgeon with a lower susceptibility of chub compared to other
411 species (Flammarion *et al.*, 2000; Minier *et al.*, 2000; Van Aerle *et al.*, 2001; Jobling *et al.*,
412 2002; Bjerregaard *et al.*, 2006; Randak *et al.*, 2009). Indeed, in our study, prevalence of
413 intersex fish did not allow for discrimination between unpolluted and polluted sites.

414 Brain AA of males and females chub from the Drôme river were of the same order as brain
415 AA measured in other wild fish species such as perch and roach (Noaksson *et al.*, 2001) and
416 bream (Hecker *et al.*, 2007). In our study, the brain AA fluctuated with the stage of maturity
417 of the gonads (histological examination). Indeed, brain AA of male and female increased with

418 maturation and when GSI increased during the reproductive period. Such fluctuations in brain
419 AA during the reproductive cycle were also observed in goldfish, sea bass, rainbow trout,
420 perch and roach suggesting an increase need of estrogens in the brain during reproduction
421 (Pasmanik and Callard, 1988; Noaksson *et al.*, 2001; Gonzalez and Piferrer, 2003; Hinfray *et*
422 *al.*, 2006a). It has been suggested that AA (and production of estrogens) in the male brain
423 during the reproductive period could be associated with sexual behavior of fish (Schlinger *et*
424 *al.*, 1999; Hallgren *et al.*, 2006). In males and females, brain AA may also be linked to the
425 secretion of gonadotropin hormones known to be involved in gonadal growth and maturation,
426 and spermiation/ovulation (Rosenfeld *et al.*, 2007). Nonetheless, these brain AA fluctuations
427 during the reproductive cycle should be kept in mind to determine the optimal time-point for
428 employing this parameter as a biomarker of endocrine disruption.

429 **The contaminated sites**

430 Among the contaminated sites of our study, the most impacted was the Jalle d'Eysines River.
431 Males from the Jalle d'Eysines River exhibited statistically significantly induced plasmatic
432 VTG levels compared to males from the reference site. Contamination of the Jalle d'Eysines
433 River by steroidal hormones have previously been shown (Labadie and Budzinski, 2005). The
434 authors showed that levels of estrogens in this river in 2003-2004 could reach 30 ng/L for
435 estrone, 1.2 ng/L for estradiol and 1 ng/L for estriol. Here we provide evidence that fish from
436 this site were affected by xeno-estrogens and it would be advisable to determine whether
437 these steroidal estrogens are responsible for the VTG inductions. In both male and female
438 chub from the Jalle d'Eysines River, an arrest of the gonadal development was observed.
439 Females from this site exhibited lower GSI compared to females from the Drôme River in
440 both seasons. Histological examination of these fish revealed that more than 70 % of the
441 females were at the primary oocytes stage during summer and fall. Concerning males of the
442 Jalle d'Eysines River, a great proportion (70%) were at the spermatogonies stage in the

443 summer, as reflected by the lower GSI and the lower brain AA observed, compared to those
444 of males from the Drôme river. As previously shown for zebrafish and fathead minnow,
445 laboratory exposure to estrogenic substances lead to reduction in GSI and inhibition of gonad
446 development (Jobling *et al.*, 1996; Miles-Richardson *et al.*, 1999; Fenske *et al.*, 2005). Thus,
447 our results on VTG and gonads development are consistent with an estrogenic exposure of
448 fish in the Jalle d'Eysines River.

449 Given the up-regulation of brain AA by exposure to EE2 (Table 2), an increase of brain AA
450 would have been expected in fish from the Jalle d'Eysines river. The absence of brain AA
451 increase might be due to xeno-estrogens concentrations in the surface water of the Jalle
452 d'Eysines River that are too low to induce brain AA. Nevertheless, all chub used for the
453 laboratory EE2 exposure were at early reproductive stages (primary oocyte and
454 spermatogonia stages) with basal brain AA lower than that of the chub caught during the
455 summer and the fall in the Jalle d'Eysines River, and thus probably allowing greater
456 inducibility of brain AA. Moreover, males caught in the summer in the Jalle d'Eysines River
457 exhibited lower brain AA than fish from the reference site. This is well in agreement with the
458 predominance of males at early stages of gonad development, for which brain AA are low
459 compared to later stages (Figure 4). Indeed, when brain AA of males was classified according
460 to the gonad stage of development, no difference was observed between males from the Jalle
461 d'Eysines River and those from the reference site (data not shown). All these results indicate
462 that the inhibition of male brain AA in summer in the Jalle d'Eysines River reflects the arrest
463 of gonad development instead of a direct effect on the aromatase expression/activity,
464 demonstrating that brain AA perturbations could be indicative of a reproductive disorder.

465 In the Nonette and the Lez rivers, chub brain AA was not affected at any season. On the
466 contrary, brain AA of males caught in summer from the Rhône River was lower than those of
467 males from the Drôme River at the same season. However, only males exhibited lower brain

468 AA, not females. Compared to males from the Drôme River, a great proportion of males from
469 the Rhône River were at the spermatogonies or spermatocytes stages in summer, suggesting a
470 delay in testis maturation. Nevertheless, it should be noted that fish from the Rhône River
471 were caught mid-May while fish from the Drôme River were caught mid-June, thus
472 introducing a potential bias. As we have previously shown, brain AA was lower in males at
473 the spermatogonies and spermatocytes stages compared to that of males at the spermatozooids
474 stage. Even if the most likely explanation for having lower brain AA in males from the Rhône
475 river might be related to the testis maturation stage of these fish, the possibility that fish were
476 exposed to aromatase inhibiting substances could not be dismissed. The presence of
477 aromatase inhibiting compounds in surface sediments extracts of the Rhône, the Jalle
478 d'Eysines and the Nonette rivers was demonstrated by using an in vitro aromatase assay
479 previously developed in the laboratory (Hinfray *et al.*, 2006a). Pesticides (more particularly
480 imidazole-like pesticides), flavonoids or PBDEs have been shown to inhibit AA in vitro in
481 different microsomal systems (Pelissero *et al.*, 1996; Le Bail *et al.*, 2000; Hinfray *et al.*,
482 2006a; Canton *et al.*, 2008). However, the precise nature of the contaminants present in
483 sediments and their potential bioavailability to fish are not known.

484 Previous studies also showed *in vivo* perturbations of the endocrine system including
485 inhibitions of brain AA in wild fish living in contaminated areas. In Sweden, a great number
486 of wild female perch (Perca fluviatilis) exposed to effluents from a public refuse dump were
487 showed to be sexually immature and this was associated with an inhibition of brain AA,
488 decreased GSI and reduced levels of steroids (Noaksson *et al.*, 2001). Similar observations
489 were made in bream (Abramis brama) where fish exposed to various industrial effluents
490 (including (agro)chemical and petrochemical plants) presented inhibited brain AA, decreased
491 GSI, and lower VTG and steroids concentrations (Hecker *et al.*, 2007). In our study, some
492 males in the Nonette and the Lez rivers presented induction of plasmatic VTG concentrations

493 as compared to males from the reference site suggesting a slight exposure of fish to estrogenic
494 substances. However, there was a large variability between males collected in one site since
495 VTG concentrations varied from non detectable up to 49 µg/ml. Such great intra-site
496 variability in plasmatic VTG concentrations was previously observed by Jobling *et al.* (1998)
497 and Flammarion *et al.* (2000) in studies on roach and chub respectively.

498 In the Nonette River, chub also exhibited strong EROD activity inductions in the two seasons.
499 EROD activities are known to respond to AhR agonists such as dioxins, PCBs, PAHs and
500 some pesticides (Whyte *et al.*, 2000). Interestingly, very strong dioxin-like activity was found
501 in sediment extracts of this site in in vitro assays using PLHC-1 cells (data not shown),
502 indicating the presence of AhR agonists in the water and sediments of the Nonette river even
503 though the precise nature of the substances was not determined. In fish, AhR agonistic
504 compounds such as dioxins, PAHs or beta-naphtoflavone can negatively affect the estrogen-
505 dependent genes expression in the liver, such as ER α and VTG, both in vitro and in vivo
506 (Navas and Segner, 2000, Bemanian et al., 2004, Aubry et al. 2005). The negative interaction
507 of AhR agonistic compounds on the estrogenic signaling pathway likely explain the
508 significant negative correlation between EROD activity and VTG synthesis ($R^2=0.565$;
509 $p<0.05$) which was found in fish from the Nonette River. As a consequence, the presence of
510 AhR agonistic compounds at this site might have led to an under-estimation of the estrogenic
511 contamination level due to interfering effects of AhR agonists also present at this site.

512 Previous field studies in France mainly focused on VTG as marker of endocrine disruption
513 (Flammarion *et al.*, 2000; Sanchez *et al.*, 2008). However, evidence shows that several
514 chemicals (alone or in mixture) can act at multiple targets to disrupt physiological functions in
515 fish. Indeed, in fish from the Jalle d'Eysines River, we showed at the same time perturbations
516 of plasmatic VTG synthesis, of in vivo brain AA and alterations of gonads development.
517 Considering the complex exposure scenario in field studies it seems difficult to assess

518 endocrine perturbations with a unique biomarker. Thus, more comprehensive
519 molecular/biochemical approaches are needed to identify pathways of endocrine disruption.

520

521 Conclusion

522

523 This study provides evidence of endocrine disruption in fish from French rivers. The
524 biomarkers used in our study allowed for the detection of perturbations in diverse tissues
525 (liver, brain, gonads) and of EDCs in sediments. In the four contaminated sites, at least one
526 biomarker was disturbed, suggesting the contamination of fish and/or sediments and
527 underlining the importance of assessing effects on different physiological targets. However, it
528 remains to determine which substances are responsible for the effects observed. For that
529 purpose, a combination of biotesting, fractionation procedures and chemical analytical
530 methods, also named Effect Directed Analysis, could be used (Brack, 2003). Moreover,
531 aromatase proved to be a promising biomarker of endocrine disruption in field studies, by
532 combining in vitro and in vivo measurements to detect contamination by aromatase inhibiting
533 substances in sediments and fish. Finally, it could be interesting to use this multiparametric
534 approach to assess endocrine disruption in a larger number of sites.

535

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537

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543

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