1   **Multibiomarker approach to fipronil exposure in the fish *Dicentrarchus labrax***

2   **under two temperature regimes**

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Abstract

Fipronil is a phenylpyrazole insecticide widely used to control pests in agriculture even though evidence of harmful side effects in non-target species has been reported. A comprehensive study on the effects of dietary administration of Regent®800WG (80% fipronil) in European sea bass juveniles was carried out under two temperature regimes: a) natural conditions, and b) 3 ºC above the natural temperature (an increase predicted for the NW Mediterranean by the end of this century). Fipronil was added to the fish food (10 mg fipronil /Kg feed) and the effects were studied at several time points including right before administration, 7 and 14 days after daily fipronil feed and one-week after the insecticide withdrawal from the diet (depuration period). A wide array of physiological and metabolic biomarkers including feeding rate, general condition indices, plasma and epidermal mucus metabolites, immune response, osmoregulation, detoxification and oxidative-stress markers and digestive enzymes were assessed. General linear models and principal component analyses indicated that regardless of water temperature, fipronil resulted in a significant alteration of several of the above listed biomarkers. Among them, glucose and lactate levels increased in plasma and decreased in epidermal mucus as indicators of a stress response. Similarly, a depletion in catalase activity and higher lipid peroxidation in liver of fipronil-exposed fish were also indicative of an oxidative-stress condition. Fipronil induced a time dependent inhibition of Cytochrome P450-related activities and an inhibition of phase II glutathione-S-transferase. Moreover, fipronil administration was able to reduce the hypo-osmoregulatory capability as shown by the increase of plasmatic osmolality and altered several digestive enzymes including trypsin, lipase, alpha amylase and maltase. Finally, analyses in bile and muscle confirmed the rapid clearance of fipronil but the persistence of the metabolite fipronil-sulfone in bile even after the 7-day depuration

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52 period. Altogether, the results reveal a notable impact of this compound on the

53 physiological condition of the European sea bass. The results should be considered in

54 future environmental risk assessment studies since fipronil could be hazardous to fish

55 species, particularly those inhabiting estuarine ecosystems exposed to the discharge of

56 agriculture runoffs where this pesticide is mainly used.

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58 **Keywords**: fipronil, sea bass, biomarkers, CYP metabolism, oxidative stress, climate

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1. Introduction

The phenylpyrazole fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-(trifluoromethylsulfonyl)pyrazole-3-carbonitrile) has been classified as moderately hazardous (Class II) by the World Health Organisation (WHO, 2009). It is one of the most used broad-spectrum insecticides in crops worldwide, being even effective against pests resistant to pyrethroids, organophosphates and carbamate insecticides (Simon-Delso et al., 2015). In Europe, fipronil is mainly used in crops of maize, rice and in sunflower seed treatment. However, its use in agriculture was severely restricted by the European Union in 2013 (Comission Implementing Regulation (EU) Nº 781/2013) due to its high acute toxicity for honeybees (European Food Safety Authority EFSA, 2013). There is strong evidence that soils, aquatic systems and plants in agricultural environments and their neighbouring areas are contaminated with fipronil and other fipronil-related substances (US Environmental Protection Agency, 1996; Bonmatin et al., 2015). Nevertheless, Spain, the largest fipronil end-user on sunflower crops in Europe, is reluctant to adhere to the European directive alluding to the existence few on-site studies evidencing its toxicity.

Fipronil and its main metabolites are toxic to non-target aquatic species (Schlenk et al., 2001; Stefani Margarido et al., 2013; Gripp et al., 2017). This compound exerts its insecticidal activity by binding to the gamma-aminobutyric acid (GABA) receptors and acting as a non-competitive blocker of GABA-gated chloride channels in the central nervous system, inducing neuronal hyperexcitation, paralysis and death (Simon-Delso et al., 2015; Huang et al., 2019). Although fipronil is generally more toxic to invertebrates than to vertebrates, due to differential affinity towards target receptors, a recent study on bighead carp (*Hypophthalmichthys nobilis*) showed that the affinity of this chemical to fish GABA receptors is similar to that found in insects, suggesting that it could also be

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102 highly toxic to fish (Zhang et al., 2018). Besides this, its main degradation products,

103 which include fipronil-sulfone, fipronil-sulfide and fipronil-desulfinyl, are less specific

104 than the parent compound, display higher insecticidal activity and also account for

105 toxicity in vertebrates (Hainzl et al., 1998; Zhao et al., 2005; Lu et al., 2010; Gupta,

106 2014; Gripp et al., 2017).

107 Human activities, including the input of pesticides into the environment, have been

108 considered as the main cause for the present world climate change scenario (CC)

109 (Hansen et al., 2006). Current consensus alerts that significant temperature increases,

110 acidification and greater salinity fluctuations of marine water bodies will occur around

111 the globe in the upcoming decades (IPCC, 2014). These changes can exert a direct

112 impact on the physiology of marine poikilotherms (Makrinos and Bowden, 2016;

113 Boltana et al., 2017; Navarro et al., 2019). On the other hand, indirect effects caused by

114 CC in marine fish are still poorly known, especially those related to their potential

115 interaction with foreign chemicals (Schiedek et al., 2007; Hooper et al., 2013). For

116 instance, changes in physical conditions and chemical exposure can act synergistically

117 magnifying the consequences of such exposures in aquatic organisms, since the former

118 can imply changes in the availability and action of chemicals (Sokolova and Lannig,

119 2008; Jacquin et al., 2019). The Mediterranean region is especially sensitive to the

120 alterations induced by CC due to its particular characteristics, such as small size,

121 relatively shallow average water depth, oligotrophy and high biological diversity,

122 among others (Calvo et al., 2011).

123 The European sea bass, *Dicentrarchus labrax* (Linnaeus, 1758) (FAO, 2005) is one of

124 the most appreciated cultured fish species in the Mediterranean. Concerns are raised

125 regarding its physiology and reproduction that could be compromised by the

126 temperature increases predicted in a CC scenario (Almeida et al., 2015). Specifically,

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127 changes in water temperature are known to adversely affect a wide number of biological

128 functions in this species including sex ratios, reproduction, growth, immune response,

129 osmoregulatory capacity, xenobiotic biotransformation and antioxidant defences, among

130 others, making the fish more vulnerable to additional stressors (Almeida et al., 2015;

131 Samaras et al., 2018). Furthermore, metabolic alterations in muscle, liver and brain in

132 response to a 4 ºC increase were enhanced after exposure to methylmercury in the

133 European sea bass, raising the possibility for a synergistic effect between both stressors

134 (Maulvault et al., 2017).

135 To the best of our knowledge, no studies have assessed potential toxic effects of fipronil

136 in *D. labrax*. However, this insecticide is known to act as endocrine disrupter in several

137 other fish (Mnif et al., 2011; Bencic et al., 2013; Sun et al., 2014), to induce oxidative-

138 stress due to reactive oxygen species (ROS) generation, and to interfere with a number

139 of isoenzymes of the cytochrome P450 (CYPs) family, a main hepatic

140 biotransformation route of this compound in different vertebrates (Wang et al., 2016).

141 The aim of this study was to evaluate bioaccumulation, biotransformation and

142 alterations in key physiological pathways of European sea bass after fipronil exposure

143 in an environmentally-realistic scenario of temperature increase predicted for the NW

144 Mediterranean region. The effects of fipronil dietary administration were assessed using

145 a wide array of biomarkers encompassing several physiological and detoxification

146 endpoints in different tissues and in two conservative matrices (i.e. plasma and skin

147 mucus) in an effort to use them as non-lethal indicators of the effects of this pesticide in

148 animal experimentation.

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150 **2. Material and Methods**

151 *2.1. Experimental design*

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152 Juvenile European sea bass (8 months old) were obtained from the Institute of Research

153 and Technology Food and Agriculture (IRTA, Sant Carles de la Ràpita, Spain). Fish

154 were transported and maintained at the Experimental Aquaria facilities (ZAE) of the

155 Institute of Marine Sciences (ICM-CSIC, Barcelona, Spain). Prior to the experiment,

156 fish were acclimated for a two-week period in a 2,500 L round fiberglass tank

157 containing filtered sea-water (sterilized sand filter 50 μm) under natural conditions of

158 temperature and with a water full-renovation rate of 24 times per day. Fish were fed

159 daily *ad libitum* with commercial pellets (L-4 Optibass 2P, Skretting, Spain). After an

160 initial two-week acclimation to lab conditions, fish were randomly assigned to four 600

161 L round fiberglass tanks (19−20 individuals per tank). Two of them were reared at

162 natural water temperature (T ≈ 13 ºC) and the other two at 3 ºC above the natural

163 temperature (T ≈ 16 ºC). The new experimental temperature was gradually attained at

164 an increasing rate of 1 °C per day and fish were acclimated to these new conditions for

165 two additional weeks. Then, fish were fed a diet containing Regent®800WG (80%

166 fipronil) at a concentration of active ingredient of 10 mg fipronil/Kg feed, which was

167 prepared following the alcohol evaporation method adapted for sea bass (Blázquez et

168 al., 1995; Blanco et al., 2016). Briefly, a monolayer of pelleted dry feed was carefully

169 sprayed with the insecticide dissolved in 15 ml ethanol and the solvent was allowed to

170 evaporate completely at room temperature and kept stored at 4 ºC until used. Fish were

171 sampled just before the start of the experimental diet (t0) and considered as control, and

172 after 7 (t7), and 14 (t14) days of fipronil administration. At this point (t14), fipronil

173 treatment finished and fish were fed with non-spiked commercial feed (depuration

174 period) for an extra week completing 21 days from the start of the experiment (t21).

175 Throughout the experiment, total feed consumption was quantified in each tank by

176 initially weighing the amount before manual feeding and, when fish stopped feeding,

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177 weighing the remaining amount; consumption was then calculated by weight difference.

178 During the experiment, values (mean ± standard deviation) of physical water parameters

179 were: Temperature = 13.37 ± 0.23 for the groups reared at natural temperature and

180 16.55 ± 0.44 ºC for those reared at +3 ºC. Other water parameters ranged as follows:

181 dissolved O_2 = 6.66 ± 0.32 and 6.59 ± 0.47 mg/L (81% and 85% saturation,

182 respectively); pH = 7.73 ± 0.35 and 7.64 ± 0.31; salinity = 37.78 ± 0.12 and 37.93 ±

183 0.27 psu, for the 13 ºC and 16 ºC groups, respectively. During the experiment, fish were

184 reared under natural photoperiod corresponding to 10 h light:14 h dark.

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186 *2.2. Fish Sampling*

187 Eight fish were sampled for each temperature regime (4 fish / replicate tank) just prior

188 to the beginning of the exposure period (t0), after 7 (t7) and 14 (t14) days of fipronil

189 administration, and after 7 days of depuration (t21). Fish were fasted for 48 h before

190 each sampling time. Fish were anesthetized with 0.2 % 2-phenoxyethanol (Sigma-

191 Aldrich, St. Louis, MO, USA), measured (standard length: SL) and weighed (total body

192 weight: BW). Epidermal mucus was collected on sterile glass slides from the over-

193 lateral line in caudal direction with especial care to avoid contamination with blood

194 and/or urogenital and intestinal excretions (Fernandez-Alacid et al., 2018). Slides were

195 gently wiped along both sides of the animal twice or three times, and mucus was

196 carefully kept in a 1.5 mL sterile tube, snap frozen in liquid nitrogen and stored at -80ºC

197 until use. About 1 ml of blood was withdrawn from the caudal vein using heparinized

198 syringes and kept on ice until centrifugation. Fish were sacrificed by severing their

199 spinal cord, eviscerated, weighed (EW) and the weight of liver, gonads and visceral fat

200 recorded. Organs/tissues, including liver, bile, gonads, digestive tract, kidney and a

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201 portion of axial muscle were collected and immediately frozen in liquid nitrogen and

202 kept at –80 ºC for further analyses.

203 Muscle and bile samples were used for chemical analyses while plasma, skin mucus,

204 liver, kidney, digestive tract and also muscle samples were used to assess a

205 comprehensive set of biomarkers reflecting different aspects of sea bass physiology and

206 metabolism (see sections below).

207 Fish were reared and sacrificed according to the Spanish regulations (RDL 53/2013),

208 and the European Directive concerning the protection of vertebrates used for

209 experimental and other scientific purposes (2010/63/EU). Procedures used were

210 approved by the ethics committee of the Local Government of Catalonia and were given

211 the reference FUE-2018-00813667. All steps were aimed to minimise animal suffering.

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213 *2.3. Tissue preparation for biochemical analyses*

214 *2.3.1. Plasma and skin mucus*

215 Plasma was obtained by blood centrifugation (eppendorf 5417R model) at 3,000 ×g for

216 15 min at 4 ºC). Mucus was homogenized using a sterile Teflon implement and

217 centrifuged at 14,000 ×g for 15 min at 4 ºC. Plasma and mucus supernatants were

218 aliquoted and stored at –80 ºC for further metabolite and biochemical analyses.

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220 *2.3.2. Muscle*

221 A portion of muscle (around 0.4 g) was homogenized in ice-cold buffer phosphate (50

222 mM pH 7.4) containing 1mM ethylenediaminetetraacetic acid (EDTA) in a 1:5 (w:v)

223 ratio using a Polytron® homogeniser. Homogenates were centrifuged at 10,000 ×g for

224 30 min at 4 ºC to obtain the S10 fraction. The supernatant was aliquoted and stored at –

225 80 ºC for further biochemical determinations.

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2.3.3. Liver

228 About 1.5 g of each liver were homogenized in ice-cold buffer phosphate (100 mM pH

229 7.4) containing 150 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM phenanthroline, 0.1

230 mg/mL trypsin inhibitor and 1 mM EDTA in a 1:4 (w:v) ratio using a Polytron®

231 blender. Homogenates were centrifuged at 10,000 ×g for 30 min at 4 °C to obtain the

232 S10 fraction, of which 1 mL was withdrawn while the rest was further homogenised at

233 100,000 ×g for 60 min at 4 °C to obtain microsomal and cytosolic fractions.

234 Microsomal pellets were dissolved in the above-described homogenization buffer, also

235 containing 20 % glycerol in a 2:1 (w:v) ratio (Crespo and Solé, 2016). S10, microsomal

236 and cytosol fractions were aliquoted and stored at −80 ºC for further biochemical

237 determinations.

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2.3.4. Kidney

240 About 0.05–0.1 g of each individual kidney were homogenised in ice-cold buffer (pH

241 7.3) containing 150 mM reagent-grade sucrose, 50 mM imidazole and 10 mM

242 Na_2EDTA in a 1:15 (w:v) ratio using a Polytron® blender. Homogenates were

243 centrifuged at 5,000 ×g for 2 min at 4 ºC. The resulting supernatant was aliquoted and

244 stored at −80 ºC for osmoregulation and enzymatic measures. A more detailed

245 description is given in González-Mira et al. (2018).

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2.3.5. Digestive tract

248 The intestines were divided into anterior and posterior regions of equal length and in

249 each of them, pancreatic (trypsin, chymotrypsin, bile salt activated lipase and alpha-

250 amylase) and intestinal brush border (BB) enzymatic activities (alkaline phosphatase,

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251 aminopeptidase N and maltase) were quantified. Further methodological details can be

252 found elsewhere for pancreatic enzymes (Gisbert et al. 2009) and intestinal enzymes

253 (Gisbert et al. 2018). The activity of non-specific esterases was also determined in the

254 pancreatic fraction. Intestines from t7 group were discarded from the study because in

255 this case fish were fasted only for 24 h, as opposed to 48 h in the other groups,

256 something that could affect the activity of digestive enzymes.

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258 *2.4. Biochemical analyses*

259 All reactions were carried out in triplicate at 25 ºC, except for CYPs, UDPGT and

260 digestive enzymes determinations, which were measured at 30 ºC on a Tecan™Infinite

261 M200 spectrophotometer.

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263 *2.4.1. Plasmatic and skin mucus metabolites and lysozyme determination*

264 Glucose and lactate content in plasma and skin mucus (expressed as μg/mL) were

265 determined by enzymatic colorimetric kit tests GOD-POD glucose (Ref: 41011) and

266 LO-POD lactate (Ref: 1001330), from SPINREACT® (Spain), according to the

267 methodology described in Fernández-Alacid et al. (2018).

268 Plasmatic ammonia (expressed as μmol/L) was analyzed using a commercial kit by

269 SPINREACT®, and (Ref: 1001410).

270 Lysozyme activity in plasma (expressed as units (U)/ mg of total plasmatic protein) was

271 measured according to the turbidimetric method described by Parry et al. (1965) with

272 some modifications. Briefly, 100 μL of plasma diluted in a 1:2 ratio with 10 mM PBS

273 pH 6.2 were placed in flat-bottomed 96-well plates. To each well, 100 μL of freeze-

274 dried *Micrococcus lysodeikticus* (0.3 mg/ml, Sigma) were added as lysozyme substrate.

275 The absorbance ($\lambda = 450$ nm) was measured at the beginning and after 15 min. Units of

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276   lysozyme present in plasma were obtained from a standard curve built with chicken egg

277   white lysozyme (HEWL, Sigma).

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279   *2.4.2. Anaerobic metabolism*

280   Lactate dehydrogenase (LDH) activity was measured in the S10 fraction of the liver

281   following adaptation of the Vassault (1983) method using NADH (200 μM) and

282   pyruvate (1 mM) as final well concentrations. Reading was done at $\lambda = 340$ nm for 5

283   min. LDH activity was expressed as nmol/min/mg total protein.

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285   *2.4.3. Oxidative-stress parameters*

286   Activities of the antioxidant enzymes catalase (CAT), total glutathione peroxidase

287   (GPX) and glutathione reductase (GR) were determined in the liver cytosolic fraction.

288   CAT activity was measured as a decrease in absorbance at $\lambda = 240$ nm using H_2O_2 (50

289   mM) as substrate; GPX and GR used cumene hydroperoxide (CHP, 0.625 mM) and

290   oxidized glutathione (GSSG, 0.9 mM) as respective substrates and NADPH as cofactor

291   in both assays at $\lambda = 340$ nm. Lipid peroxidation levels (LPO) were quantified in muscle

292   and in S10 liver fraction using a colorimetric method with 1-methyl-2-phenylindole.

293   Quantification, with respect to the standard solution 1,1,3,3-tetramethoxypropane, was

294   made at $\lambda = 586$ nm. CAT activity was expressed as μmol/min/mg total protein and GR

295   and GPX activities as nmol/min/mg total protein and LPO levels as nmol MDA

296   (malondialdehyde)/g wet weight.

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298   *2.4.4. Conjugation enzymes*

299   Glutathione *S*-transferase (GST) determination was performed in the liver cytosolic

300   fraction according to the method of Habig et al. (1974) using 1 mM GSH as substrate at

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301 λ = 340 nm. Uridine diphosphate glucuronyltransferase (UDPGT) activity was

302 measured in liver microsomes according to the method of Collier et al. (2000) using

303 methyl umbelliferone (MU, 0.1 mM) as substrate in the fluorometric mode (EX/EM

304 355/460). Both activities were expressed as nmol/min/mg total protein.

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306 *2.4.5. CYP components and reductases*

307 Catalytic activities of hepatic CYPs were determined in the microsomal liver fraction

308 using six fluorometric substrates: 7-ethoxyresorufin (ER), 7-benzyloxyresorufin (BR),

309 7-methoxyresorufin (MR), 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 3-cyano-7-

310 ethoxycoumarin (CEC) and 7-ethoxycoumarin (EC). Assay conditions were based on

311 the method by Solé et al. (2012). Briefly, microsomes (10 µL) were incubated for 10

312 min at 30 ºC and the metabolite formed was recorded at its specific wavelength (Smith

313 and Wilson, 2010). A calibration curve for each specific metabolite was done (range 0–

314 160 nM). CYPs assays were run in 100 mM phosphate buffer pH 7.4, except for ECOD

315 determination, which was done in 100 mM Tris buffer pH 7.4. Activities were

316 expressed in pmol/min/mg total protein.

317 Microsomal reductases, NAD(P)H- cytochrome c reductases and NADH- ferricyanide

318 reductase activities, were measured by the increase in absorbance at λ = 550 nm and the

319 decrease in absorbance at λ = 420 nm, respectively (Solé and Livingstone, 2005). Assay

320 conditions were: 50 mM Tris-HCl buffer pH 7.6, 1 mM KCN, 0.26 mM NAD(P)H, and

321 60 µM cytochrome c or 0.2 mM potassium ferricyanide. Sample volumes were: 10 µL

322 microsomal fraction for NADPH- and 15 µL for NADH-dependent reductases. Results

323 are expressed in nmol/min/mg total protein.

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325 *2.4.6. Osmoregulation*

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326   Activity of Na^+/K^+-ATPase was determined in the head kidney of fish following Zaugg

327   (1982) method with modifications (González-Mira et al., 2018). Sample absorbance was

328   measured at $\lambda = 750$ nm after 30 min incubation. Na^+/K^+-ATPase activities were

329   expressed as μmol ATP hydrolysed/mg total protein/hour. Plasmatic osmolality was

330   measured with the aid of a Fiske® 210 Micro-Sample Osmometer using 20 μL plasma

331   and expressed in mosm/Kg H_2O.

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333   *2.4.7. Digestive enzymes*

334   The methods used for enzyme quantification are briefly described as follows: trypsin

335   and chymotrypsin activities, the two main pancreatic alkaline proteases, were assayed

336   using, respectively, N-benzoyl-DL-arginine p-nitroanilide (BAPNA) (Holm et al., 1988)

337   and Succinyl-L-Ala-Ala-Pro-L-Phenylalanine p-nitroanilide (SAAPNA) (Erlanger et

338   al., 1961). Alpha-amylase activity was estimated using 2-chloro-p-nitrophenyl-α-D-

339   maltotrioside as substrate (Lorentz et al., 1999). The activities of bile-salt-activated

340   lipase and non-specific esterases were measured using p-nitrophenyl myristate (Iijima et

341   al., 1998) and p-nitrophenyl acetate (Hosokawa and Satoh, 2005) as respective

342   substrates. The activity of the alkaline phosphatase was determined using 4-

343   nitrophenylphosphate (Bessey et al., 1946), aminopeptidase N activity was determined

344   using L-leucine p-nitroanilide (Maroux et al., 1973) and maltase activity was

345   determined using d(+)-maltose (Dahkqvist, 1970) as substrates. All enzymatic activities

346   were expressed as specific units (mU/mg total protein).

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348   *2.4.8. Protein determination*

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349 Total protein content of all samples was determined by the Bradford method (1976)

350 using the Bio-Rad Protein Assay reagent and bovine serum albumin (BSA; 0.05-1

351 mg/mL) as standard. The absorbance was read at $\lambda = 595$ nm.

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353 *2.5. Chemical analyses*

354 *2.5.1. Bile*

355 Bile glands from group t7 were mostly empty (as described in digestive enzyme

356 measures), and could not be used for chemical analysis. A more detailed methodology

357 (adapted from Aceña et al. (2017)), as well as reference standards and solvent solutions

358 characteristics and a description for fipronil and its metabolites quantification, is

359 reported as electronic supplementary material (ESM).

360 All analyses were performed using a SCIEX ExionLC™ AD system coupled to a hybrid

361 SCIEX X500R QTOF system (Sciex, Redwood City, CA, U.S.) equipped with a Turbo

362 V™ source and Electrospray Ionization (ESI).

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364 *2.5.2. Muscle*

365 Analysis of fipronil in muscle was based on the use of the commercial brand

366 Regent®800WG (80% fipronil) as standard and following the solid phase QuEChERS

367 extraction method and gas chromatography-electron impact (GC-EI) detection at the

368 Scientific and Technological Centres of the University of Barcelona (CCiTUB) that

369 holds the quality standard ISO 9001:2015. A more detailed description of the analytical

370 procedure is provided as ESM.

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372 *2.6. Data analyses*

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373 Fish hepatosomatic index (HSI) and gonadosomatic index (GSI) were calculated as

374 (liver weight/BW) × 100 and (gonad weight/BW) × 100, respectively. Fish condition

375 was assessed by Fulton's condition factor (CF), calculated as $(BW/TL^3) \times 100$.

376 Daily feed intake was measured per tank and food consumption calculated in relation to

377 the total number of fish per tank. For each mucus sample, the ratios of glucose/lactate,

378 glucose/protein and lactate/protein were calculated.

379 A detailed explanation of statistical procedures is provided as ESM. In short,

380 relationships among fish biological and some biochemical variables and their

381 interactions were tested by Pearson' or Spearman' rank correlations (continuous

382 variables) and by Student's t-tests or Mann-Whitney U-tests (sex-related differences).

383 For digestive enzymes, differences between anterior and posterior parts of the digestive

384 tract were tested using Wilcoxon pairwise tests with repeated measurements.

385 Possible effects of treatment duration and temperature were tested by general or

386 generalized linear models (GLMs/GZMs) followed by Student's t-test/Mann-Whitney

387 U-test and one-way ANOVA/Kruskal-Wallis tests. A permutation multivariate analysis

388 (PERMANOVA) and a principal component analysis (PCA) were also carried out in

389 order to assess a global biochemical response to temperature and treatment duration In

390 all cases, significant differences were set at $p < 0.05$.

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392 **3. Results**

393 *3.1. Biometric parameters and general condition indices*

394 Mean values for fish biometric data and general condition indices were fairly uniform

395 across experimental groups (Table 1). Most individuals were immature, as evidenced by

396 low GSI values in both sexes. Sex ratios were generally skewed, with more females

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397   than males in most groups. Significant increases of SL and BW with time were

398   observed ($\chi^2 = 10.589$, $p = 0.014$ and $\chi^2 = 8.613$, $p = 0.035$, respectively).

399   Direct correlations among biometric data and condition indices were found in most

400   cases, with visceral fat weight showing positive associations with all other biological

401   variables ($r_p = 0.299–0.692$, $p = 0.018 – < 0.001$), in a similar way as BW (only non-

402   significant correlation to HSI) ($r_p = 0.268–938$, $p = 0.032 – < 0.001$). SL and GSI were

403   also positively correlated ($r_p = 0.483$, $p < 0.001$), as well as CF and HSI ($r_p = 0.457$, $p <$

404   0.001). Body weight, GSI and visceral fat weight displayed higher values in females

405   than in males ($t = 2.690–6.252$, $p = 0.012– < 0.001$).

406

407   *3.2. Feed consumption*

408   Fish feeding rate was similar at the two experimental temperatures: 0.54 ± 0.20 and 0.57

409   ± 0.22 g/fish at 13 ºC and 16 ºC, respectively, and significantly increased over time ($F_{(3,}$

410    $_{24)} = 23.891$, $p < 0.001$) (Table 1).

411

412   *3.3. Biochemical analyses*

413   *3.3.1. Plasmatic and skin mucus analyses*

414   Mean levels of plasmatic glucose ranged between 110.40 ± 14.20 and 212.30 ± 22.20

415   mg/dL and lactate between 52.70 ± 5.50 and 79.30 ± 7.10 mg/dL. In mucus these values

416   were much lower with glucose ranging between 0.57 ± 0.07 and 1.24 ± 0.11 mg/dL and

417   lactate between 0.42 ± 0.08 and 1.11 ± 0.14 mg/dL, (Table S1). Some statistical

418   correlations of the different parameters studied in plasma and skin mucus and most fish

419   biometrics and condition indices are detailed in Table S2.

420   Although no effect of the rearing temperature was found, a significant increase with

421   longer fipronil exposures was observed for glucose and lactate plasma levels ($F_{(3, 59)} =$

17

422 7.098, $p < 0.001$ and $F_{(3, 53)} = 4.226$, $p = 0.009$, respectively) (Fig. 1A, B). Moreover, a

423 decrease of these biomarkers during the depuration period was observed in skin mucus

424 ($F_{(3, 59)} = 10.196$, $p < 0.001$ and $F_{(3, 59)} = 6.338$, $p = 0.001$, respectively) (Fig. 1C, D).

425 Glucose/protein and lactate/protein ratios (mean values $3.31 \pm 0.47 - 6.39 \pm 0.98$ µg/mg

426 and $2.28 \pm 0.24 - 4.59 \pm 0.37$ µg/mg, respectively) in skin mucus were not affected by

427 temperature or fipronil exposure. However, glucose/lactate ratio ($1.06 \pm 0.12 - 1.95 \pm$

428 0.29 µg/mg) was higher at 13 ºC than at 16 ºC ($t = 2.733$, $p = 0.008$) (Table S1). A

429 significant interaction between exposure time and CF was found for lactate content in

430 mucus ($F_{(3, 54)} = 6.113$, $p = 0.001$). Strong positive correlations were detected among

431 protein, glucose and lactate both in plasma ($r_p = 0.411$–0.632, $p < 0.001$) and skin

432 mucus ($r_p = 0.434$–0.706, $p < 0.001$).

433 Plasmatic lysozyme activity ranged between 7.98 ± 2.13 and 13.24 ± 2.53 U/mg total

434 protein (Table S3) and was not affected by temperature or fipronil exposure time.

435

436 *3.3.2. Anaerobic metabolism*

437 Mean LDH activity in liver ranged between 14.58 ± 1.42 and 16.25 ± 1.17

438 nmol/min/mg total protein (Table S3), with no association to fish biometric variables or

439 condition indices and it was not affected by water temperature or fipronil exposure.

440

441 *3.3.3. Oxidative-stress parameters*

442 Antioxidant enzymes GR ($5.56 \pm 1.05 - 7.98 \pm 0.84$ nmol/min/mg total protein), GPX

443 ($6.60 \pm 0.40 - 8.12 \pm 0.40$ nmol/min/mg total protein) and CAT ($63.84 \pm 10.27 - 111.12$

444 ± 68 µmol/min/mg total protein) (Table S3) were not affected by temperature, and only

445 CAT responded to fipronil exposure, decreasing after the depuration period (t21) ($F_{(3, 60)}$

18

446 = 4.792, p = 0.005) (Fig. 2A). Regarding fish biological variables, only some negative

447 associations were observed between GR and some biological traits (Table S2).

448 Mean LPO levels ranged between 2.99 ± 0.74 and 6.21 ± 1.09 nmol MDA/g ww in

449 muscle and between 10.10 ± 1.74 and 17.35 ± 1.96 nmol MDA/g ww in the S10 liver

450 fraction (Table S3). In both cases, no effect of temperature was detected but a

451 significant increase in MDA equivalents during fipronil exposure was found in liver

452 ($F_{(3, 60)}$ = 7.436, p < 0.001) (Fig. 2B).

453 Correlations among oxidative stress-related biomarkers are shown in Table 2, revealing

454 a similar trend for GR and GPX activities, while GR scaled negatively with CAT

455 activity and LPO levels in liver.

456

3.3.4. Conjugation enzymes

458 Mean GST activity values ranged between 36.45 ± 2.43 and 51.05 ± 5.80 nmol/min/mg

459 total protein, and UDPGT between 0.75 ± 0.09 and 0.82 ± 0.08 nmol/min/mg total

460 protein (Table S3). Some positive correlations were detected between conjugation

461 enzymes and biological variables, and GST activity was higher in females (Table S2).

462 Both enzymes were unaffected by temperature and GST activity was significantly

463 enhanced with increasing fipronil exposure time ($F_{(3, 60)}$ = 4.245, p = 0.009) (Fig. 2C).

464 GST activity displayed positive correlations with LPO in muscle and liver and was

465 negatively related to GR activity, while UDPGT activity was positively correlated with

466 CAT activity (Table 2).

467

3.3.5. CYP components and reductases

469 EROD (mean activity = 15.33 ± 1.31 – 25.35 ± 3.86 pmol/min/mg total protein), BROD

470 (0.47 ± 0.07 – 0.76 ± 0.11 pmol/min/mg total protein), MROD (2.34 ± 0.18 – 5.00 ±

19

471 0.71 pmol/min/mg total protein), CECOD ($19.14 \pm 1.63 - 33.45 \pm 5.34$ pmol/min/mg

472 total protein) and ECOD ($3.35 \pm 0.66 - 9.42 \pm 1.47$ pmol/min/mg total protein)

473 activities (Table S3) displayed negative correlations with fish HSI, as well as negative

474 associations between MROD and visceral fat weight and between ECOD and CF (Table

475 S2). These same formerly mentioned activities were unaffected by temperature but

476 significantly decreased after fipronil exposure ($F_{(3, 60)} = 3.046$, $p = 0.036$; $F_{(3, 60)} =$

477 4.327, $p = 0.008$; $F_{(3, 60)} = 7.743$, $p < 0.001$, $F_{(3, 60)} = 3.795$, $p = 0.015$ and $F_{(3, 47)} =$

478 3.211, $p = 0.031$, respectively) (Fig. 2D, E). By contrast, BFCOD activity (62.91 ± 5.23

479 $- 98.97 \pm 22.34$ pmol/min/mg total protein) was not affected by temperature of fipronil

480 exposure (Table S3). Strong positive correlations were found among most CYPs and

481 between them and the oxidative-stress markers GR and GPX (Table 2). By contrast,

482 negative associations were observed between GST and MROD and between UDPGT

483 and most CYPs (Table 2).

484 In relation to CYP-dependent reductases (expressed in nmol/min/mg total protein):

485 NAD(P)H Cyt c reductase ($17.07 \pm 1.22 - 22.98 \pm 2.24$) and NADH-Cyt c ($22.79 \pm$

486 $3.36 - 31.97 \pm 5.34$) and ferrycyanide reductases ($1006 \pm 95 - 1208 \pm 108$) (Table S3),

487 NADPH cyt c reductase displayed higher activity at the lowest temperature ($t = 2.575$, p

488 $= 0.012$) (Fig. 2F) and a positive weak correlation with fish visceral fat weight, and of

489 NADH Cyt c reductase, that was negatively associated to fish GSI (Table S2).

490

491 *3.3.6. Osmoregulation*

492 Kidney Na$^+$/K$^+$-ATPase mean activity ranged from 0.91 ± 0.11 to 1.94 ± 0.51 µmoles of

493 ATP hydrolysed/mg total protein/hour (Table S3), osmolality from 370.50 ± 4.69 to

494 400.50 ± 9.12 mosm/Kg H$_2$O and ammonia from 153.10 ± 9.46 to 333.62 ± 59.29

495 µmol/L (Table S1). While Na$^+$/K$^+$-ATPase activity and ammonia content did not

20

496 significantly vary as a function of temperature or experimental time, osmolality showed

497 a significant increase after fipronil exposure ($F_{(3,\ 57)}$ = 6.729, p = 0.001).

498

499 *3.3.7. Digestive enzymes*

500 Significant differences in activity between anterior and posterior intestine sections were

501 detected for the pancreatic enzymes trypsin (t14 at 13 °C; W = 36, p = 0.012) and

502 chymotrypsin (t21 at 16 °C; W = 27, p = 0.038) and the BB enzymes alkaline

503 phosphatase (t21 at 13 and 16 ºC; W = 27, p = 0.039 and W = 28, p = 0.023,

504 respectively), aminopeptidase N (t21 at 13 and 16 ºC; W = 33, p = 0.043 and W = 35, p

505 = 0.019, respectively) and maltase (t0 at 16 °C and t21 at 13 and 16 ºC; W = 21–36, p =

506 0.012–0.046). In light of these results, data from digestive enzymes for anterior and

507 posterior intestine regions were treated separately.

508 Regarding pancreatic enzymes (in mU/mg total protein) in anterior intestine: trypsin

509 (45.60 ± 3.07 − 61.20 ± 2.63), chymotrypsin (61.60 ± 15.87 − 104.70 ± 33.26), bile salt-

510 activated lipase (21.50 ± 3.73 − 42.70 ± 11.33), alpha-amylase (4.40 ± 0.92 − 10.50 ±

511 3.01) and non-specific esterases (415.60 ± 38.22 − 564.40 ± 35.19) (Table S4), lower

512 activities were observed for trypsin before fipronil exposure (t0) and after depuration

513 period (t21) ($F_{(2,\ 41)}$ = 5.193, p = 0.01) (Fig. 2G), and for lipase and alpha-amylase over

514 time ($F_{(2,\ 44)}$ = 3.435, p = 0.042 and $F_{(2,\ 45)}$ = 3.708, p = 0.033, respectively). For

515 posterior intestine: trypsin (45.70 ± 3.22 − 64.40 ± 10.03), chymotrypsin (117.30 ±

516 39.39 − 428.50 ± 158.87), lipase (24.90 ± 3.82 − 30.80 ± 3.94), alpha-amylase (3.10 ±

517 0.44 − 9.70 ± 2.48) and non-specific esterases (447.40 ± 43.69 − 587.70 ± 56.37) no

518 significant trends were detected. In relation to intestinal BB enzymes (in mU/mg total

519 protein) in anterior intestine: alkaline phosphatase (742.40 ± 116.31 − 1,177.40 ±

520 284.87), aminopeptidase N (117.30 ± 22.32 − 220.50 ± 22.80) and maltase (42.90 ±

21

521   8.63 – 250.10 ± 50.02), higher activities were found for aminopeptidase N at 13 ºC (t =

522   2.310, p = 0.026) and for maltase with time exposure ($F_{(2, 45)}$ = 34.975, p < 0.001) (Fig.

523   2H). Among the enzymes assessed in posterior intestine: alkaline phosphatase (540.80 ±

524   109.19 – 826.20 ± 175.18), aminopeptidase N (93.50 ± 11.08 – 178.90 ± 27.34) and

525   maltase (26.20 ± 2.92 – 158.20 ± 23.95), only maltase activity increased over time ($F_{(2,}$

526   $_{45)}$ = 28.828, p < 0.001).

527   Regarding to association of digestive enzymes with fish biometric variables and general

528   condition indices, few significant associations were found (Table S2) while mostly

529   positive correlations were found among intestinal enzymes (Tables S5 and S6).

530

531   *3.4. Chemical analyses on bile and muscle*

532   Concentrations of fipronil and fipronil-sulfone in bile and in muscle (only fipronil) of

533   the different experimental groups are shown in Table 3. In addition, a more detailed

534   report on chemical results is provided as ESM.

535

536   *3.5. Multivariate analyses*

537   PERMANOVA analyses showed no effect of temperature but a significant influence of

538   fipronil on fish general biochemical profile (*Pseudo-F*$_{(3, 60)}$ = 2.790, $p_{(perm)}$ = 0.0001;

539   9876 unique permutations, all pairwise comparisons significant except those comparing

540   t7 and t14, and t14 and t21).

541   Two-dimensional PCA plots represented 56.1 % of total variance on the first two

542   components, and 51.9 % of the total variance on the first and third components (Figure

543   3). These results suggest a differentiation according to the exposure time to fipronil

544   along the first axis, with samples corresponding to unexposed fish (t0) (Fig. 3A, right

545   part of the plot) clearly separated from the rest (Fig. 3A, left part of the plot), and

22

546 according to temperature along the third axis (Fig. 3B). Pearson correlations indicated

547 associations between some biochemical markers and fish groups, namely between most

548 CYP-related activities and unexposed fish (t0), between LPO levels, plasmatic

549 metabolites and osmolality and fish from t7 and t14, and between LDH, NADPH-Cyt c

550 reductase and GST and fish from t14 and t21.

551

552 **4. Discussion**

553 The present study reports, for the first time, the effects of dietary fipronil exposure on

554 several physiological parameters of the European sea bass. The use of a comprehensive

555 set of biomarkers encompassing different physiological and detoxification processes

556 allows for the assessment of the effects of this pesticide on the health and general

557 condition of an economically important cultured fish species. Moreover, the combined

558 effects of fipronil and a 3 ºC temperature increase (as predicted for the NW

559 Mediterranean region by the end of this century) constitute a novel approach to assess

560 the consequences of CC for the harmful effects of this chemical in this commercial fish

561 species widely used for human consumption.

562 Morphometric markers and condition indices remained unchanged regardless of fipronil

563 exposure or of the rearing temperature, suggesting that the 3 ºC temperature variation,

564 fipronil concentration and/or the time of exposure assessed were below threshold limits

565 to affect them. The observed increase in feeding rate over time regardless of the

566 temperature likely accounts for higher feed consumption as fish increased in size, and

567 not to fipronil exposure, since no changes occurred during depuration.

568 Despite of the wide array of biochemical markers assessed in this study, the use of

569 multivariate tools helped to infer some general patterns in their response to the different

570 experimental conditions. It appears that temperature induced changes in some metabolic

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571   parameters as shown by the segregation along the third PCA axis of fish reared at 13 ºC

572   and 16 ºC but the PERMANOVA analyses indicated that the temperature-induced

573   metabolic changes observed were not that clear. An integrated multi-biomarker

574   response to fipronil exposure, according to both multivariate analyses, showed that the

575   most prominent changes took place between unexposed (t0) and exposed (t7, t14 and

576   t21) fish. Notably, biochemical patterns after depuration (t21) clustered with those for

577   t14 fish both in PERMANOVA analysis and PCA plots, rather than with non-exposed

578   groups (t0) suggesting that the 7-days depuration period was too short to allow for the

579   full recovery of pre-exposure levels.

580   The choice of a modest temperature increase, 3 ºC with respect to the group reared at

581   natural temperature, was considered as environmentally realistic under the IPCC

582   forecasts by year 2100, although sharper increases may occur in estuarine and coastal

583   ecosystems (IPCC, 2014) inhabited by European sea bass, particularly in their juvenile

584   stage. The generalized lack of effects of this temperature increase on the assessed

585   biomarkers contrasts with other studies performed on juveniles of this species, which

586   reported behavioural, physiological and biochemical changes although under warmer

587   conditions that could account for the different results (Vinagre et al., 2012; Almeida et

588   al., 2015). Furthermore, a synergic effect between warmer conditions and fipronil

589   exposure did not occur in the present study. However, one must keep into account that a

590   more realistic simulation of CC conditions, including alterations of other abiotic

591   variables (e.g. salinity, pH), could yield a different outcome.

592   In the present study, several parameters in plasma and mucus were included as potential

593   non-lethal indicators of fish stress condition. Glucose and lactate increases in plasma

594   could be a result of the mobilisation of energetic resources induced by higher metabolic

595   demands, especially during the depuration period. In turn, the concomitant drop in

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596 mucus could be explained by the need to spare energy when energetic demands

597 increase, as described in gilthead sea bream during a 2-weeks starvation period

598 (Fernández-Alacid et al., 2018) or under chronic cold temperature conditions (Sanahuja

599 et al., 2019). Thus, plasma and mucus metabolite levels do not necessarily match under

600 chronic or sustained stress conditions (several days-weeks), as it is observed in the

601 present study and contrary to what has been reported under acute stress (hours)

602 (Fernández-Alacid et al., 2019).

603 In fish, fipronil metabolism takes place by oxidation and reduction reactions catalysed

604 by cytochrome P450-related enzymes (CYPs), which generate different fipronil

605 metabolites (mainly fipronil sulfone) (Konwick et al., 2006; Wang et al., 2016; Li et al.,

606 2018). These metabolites can be even more toxic to insects, mammals, aquatic

607 organisms and birds than the parental compound (Leghait et al., 2009; Tavares et al.,

608 2015). In the present study, this important phase I metabolic pathway was assessed by

609 using several fluorometric substrates indicative of several CYP isoforms (Smith and

610 Wilson, 2010; Solé et al., 2014) and the general electron donors NAD(P)H Cyt c and

611 NADH ferrycyanide reductases. The responses of CYP1A- and CYP2B-related

612 activities (EROD, BROD, MROD, ECOD and CECOD) showed a similar trend,

613 markedly decreasing after fipronil administration, suggesting certain overlapping

614 substrate specificity, whereas CYP3A4-associated BFCOD activity displayed the

615 opposite trend. The effects of fipronil on CYP-related activities is controversial; for

616 instance, *in vitro* studies with human hepatocytes (Das et al., 2006) suggested an

617 increase in CYP1A1-related activity at low concentrations (1 μM), and a decrease at

618 higher ones (10 and 25 μM). In the present study, ECOD activity was clearly inhibited

619 after 14 days of fipronil exposure and remained low even at the end of the depuration

620 period. This CYP-related activity was the only one responding to waterborne fipronil

25

621   exposure in the zebrafish, *Danio rerio*, showing a dose-dependent induction in several

622   tissues 24h after exposure (Wu et al., 2014). A recent study in the Caspian kutum fish,

623   *Rutilus kutum*, showed a strong correlation between *cyp1a* gene expression and different

624   antioxidant responses in several tissues, including liver (Ardeshir et al., 2018). The

625   study suggested that the increase of *cyp1a* gene expression after intraperitoneal (IP)

626   fipronil injection could be due to structural similarities between this compound and

627   some aryl hydrocarbon receptor agonists, such as halogenated hydrocarbons (Ardeshir

628   et al., 2018). In fact, fipronil transformation into more toxic metabolites fipronil-sulfide

629   and fipronil-sulfone is linked to oxidative stress (Wang et al., 2016). This is supported

630   by the present results, since CAT, the antioxidant enzyme, was inhibited after the

631   longest exposure to fipronil, supporting the notion that the production of oxyradicals

632   may overwhelm the protective capacity of this enzyme (Regoli and Giuliani, 2014).

633   Moreover, the increase of LPO levels in liver and muscle clearly confirmed a scenario

634   of oxidative damage to cell membrane lipids, most likely due to ROS generation as

635   previously suggested (Wang et al., 2016). In addition to the role of GST catalysing the

636   conjugation of glutathione with xenobiotics for detoxification purposes, other GST

637   isoforms appear implicated in the reduction of lipid hydroperoxides produced by ROS

638   (Regoli and Giuliani, 2014). Present data point at this possibility, as suggested by the

639   progressive increase in GST activity after fipronil exposure. Nonetheless, this increase

640   in antioxidant protection, as indicated by the positive correlations between GST activity

641   and LPO levels in liver and muscle was not enough to prevent the occurrence of

642   oxidised lipids even after depuration. Concordantly, several studies performed in fish

643   also reported the occurrence of oxidative-stress after fipronil administration, either

644   waterborne exposure (Clasen et al., 2012; Menezes et al., 2016; Ghazanfar et al., 2018),

645   after IP injection (Ardeshir et al., 2017a) and even considering a combination of

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646 waterborne exposure and IP injection (Ardeshir et al., 2017b). Thus, induction of

647 oxidative stress is a well-accepted consequence of fipronil exposure.

648 Since the European sea bass is a euryhaline fish that inhabits waters with broad salinity

649 gradients during its life cycle, osmoregulation constitutes a key physiological process

650 worth to be considered. In the present study, two osmoregulation-related parameters

651 were assessed. A significant increase in plasmatic osmolality evidenced a reduced

652 capability of hypo-osmoregulation in seawater after fipronil administration. However,

653 another parameter also indicative of osmoregulation capacity such as kidney Na^+/K^+

654 ATPase activity was not affected. At this stage, we can only speculate that another

655 response on this parameter might have been obtained if it had been measured in gills

656 instead of kidney, since this marker seems to display a tissue-dependent pattern of

657 activity (Vargas-Chacoff et al., 2009). Consequences for an osmoregulatory imbalance

658 after fipronil exposure are particularly significant in this species, given that during early

659 life stages it inhabits estuarine and freshwater ecosystems potentially subjected to waste

660 water discharges from nearby agricultural areas where the insecticide may be used.

661 In the present study, the consequences of fipronil exposure were also evaluated in the

662 digestive system because of the high importance of growth and energy assimilation in

663 this cultured fish species. The effects of temperature and fipronil on the activity of the

664 main digestive pancreatic and BB enzymes were evaluated in proximal and distal

665 intestinal parts since fish intestine is characterized by proximo-distal gradients of

666 hydrolases (Xiong et al., 2011; Izvekova et al., 2013). In the present study, the decrease

667 on lipase activity in bile might be due to the presence of fipronil-derived compounds in

668 bile, such as fipronil sulfone, that could have impaired lipase activity. Similarly, the

669 pyretroid insecticide deltamethrin, used in combination with fipronil (Jiang et al., 2014),

670 has been shown to inhibit lipase activity in several fish species (Simon et al., 1999;

27

671   Gunes and Yerli, 2011). Regarding the glucosidases alpha-amylase and maltase,

672   suppression of the activity of the former has been reported in several fish species

673   exposed to different pollutants, and mostly explained by a reduction in substrate affinity

674   (Filippov et al., 2013). The opposite trend was observed for maltase activity in anterior

675   and posterior intestine regions after fipronil exposure. This is in line with the random

676   effects of toxics on glucosidases ranging from inhibition to stimulation depending on

677   toxicant concentration, its interaction with other chemicals and exposure time (Filippov

678   and Golovanova, 2012; Filippov et al., 2013). Therefore, the response of glucosidases to

679   fipronil (whose effects on digestive enzymes have never been addressed before) needs

680   to be further investigated before more consistent conclusions could be drawn. Similarly,

681   the effect of organic pollutants on proteolytic activity (i.e. trypsin) seems inconsistent;

682   while it significantly increased in the present study after fipronil administration, it

683   decreased in roach (*Rutilus rutilus*) exposed to polychlorinated biphenyls (PCBs)

684   (Golovanova et al., 2011) and it was unaffected by naphthalene (a polycyclic aromatic

685   hydrocarbon) in Mozambique tilapia, *Oreochromis mossambicus*, (Kuz'mina et al.,

686   1999). Since exposure to fipronil did not result in a decrease of BB enzymes, as

687   indicative of harm to enterocytes integrity (Lalles, 2010), it seems that no damage

688   occurred to intestinal epithelium at the tested concentration.

689   Chemical analyses in muscle and bile confirmed intake and clearance of fipronil during

690   the exposure period as well as a bioaccumulation trend over time and a depuration after

691   withdraw from diet. Higher levels of the metabolite fipronil-sulfone than those of the

692   parental fipronil in fish bile at t14 and t21 confirmed a metabolisation of the insecticide

693   within a few days. Notably, in rainbow trout, *Oncorhynchus mykiss* (Konwick et al.,

694   2006) and Nile tilapia, *Oreochromis niloticus*, (Li et al., 2018), fipronil-sulfone was

695   detected as soon as one day after exposure to the parent compound, indicating its rapid

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696   biotransformation. In the present study, the parent compound fipronil was not detected

697   in the muscle and very low concentrations were quantified in bile at the end of the 7-day

698   depuration period (t21). This is in agreement with the rapid elimination reported by

699   Konwick et al. (2006), who could not detect the pesticide in trout muscle 4 days after

700   the end of the exposure. In contrast, fipronil-sulfone persisted in bile after the

701   depuration period (7 days after the end of fipronil administration) at fairly high

702   concentrations, which may be related to its affinity towards organic carbon supporting

703   the view that fatty organs, such as liver, can act as a reservoir for fipronil residues (Li et

704   al., 2018; Qu et al., 2018). Indeed, fipronil-sulfone is considered to be more toxic to

705   aquatic species than fipronil itself (EPA, 1996). This was also confirmed in the present

706   study by modulation of the activities: reduced CAT, enhanced GST and LPO

707   occurrence even at the end of the depuration period. Some studies in fish have alerted

708   for the high bioaccumulation potential of fipronil-sulfone compared to the parent

709   compound (Konwick et al., 2006; Wang et al., 2016). Moreover, our results may also be

710   suggestive of a longer persistence of fipronil-sulfone with increasing temperatures, as

711   higher concentrations of this metabolite were present in the bile of European sea bass

712   reared at 16 ºC than in those reared at 13 ºC However, more studies are needed to

713   strengthen this hypothesis. If confirmed, a potential synergistic interaction between

714   fipronil-sulfone and warmer temperatures should be taken into consideration when

715   predicting future consequences in a global warming scenario.

716

Conclusions

718   A two-week dietary administration of the pesticide fipronil induced physiological

719   responses in the European sea bass, as indicated by alterations in several markers.

720   Trends on plasma and skin mucus metabolites were indicative of an increased energy

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721 demand during fipronil exposure and after depuration. Fipronil administration also

722 caused an oxidative-stress condition that persisted even after depuration and was

723 accompanied by the modification of some phase I CYP-related activities and an increase

724 of phase II GST activity. Osmoregulation and some digestive enzymes were also altered

725 as a consequence of the pesticide administration. Chemical analyses in bile and muscle

726 confirmed intake and clearance of fipronil (faster in muscle than in liver) but persistence

727 of the metabolite fipronil-sulfone in bile even after the depuration period. Although a

728 modest temperature increase of 3 ºC did not enhance fipronil effects, the persistence of

729 fipronil-sulfone in bile at higher temperature may alert for potential synergistic effects

730 in a CC scenario.

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742 **Conflict of interest**

743 The authors of the present study declare that they have no conflict of interest.

744

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Tables

Table 1. Mean ± standard deviation for biometric measurements, general condition indices and feeding rate of European sea bass exposed to fipronil under two temperature regimes before exposure (t0) after 7 and 14 days of exposure (t7 and t14, respectively) and after a 7-day depuration period following exposure (t21). Provided values on feeding rate were calculated from food consumption reported for each group during the previous week to each sampling. N: number of individuals, F: females, M: males, SL: standard length, TW: total weight, CF: condition factor, HSI: hepatosomatic index, GSI: gonadosomatic index.

T °C	Time	N (F:M)	SL (cm)	TW (cm)	CF	HSI	GSI (F)	GSI (M)	Visceral fat (g)	Feeding rate (g/fish)
13 °C	t0	8 (2:6)	20.50 ± 2.67	125.08 ± 52.18	1.37 ± 0.08	2.08 ± 0.71	0.15 ± 0.02	0.11 ± 0.12	5.05 ± 2.41	0.42 ± 0.16
	t7	8 (6:2)	23.04 ± 0.60	174.27 ± 17.67	1.43 ± 0.19	2.24 ± 0.84	0.28 ± 0.03	0.24 ± 0.06	6.53 ± 1.63	0.46 ± 0.08
	t14	8 (6:2)	21.65 ± 1.62	145.81 ± 27.47	1.42 ± 0.10	2.32 ± 0.73	0.20 ± 0.09	0.04 ± 0.01	6.07 ± 1.44	0.49 ± 0.11
	t21	8 (4:4)	22.56 ± 0.82	164.99 ± 9.11	1.44 ± 0.11	2.72 ± 0.47	0.23 ± 0.08	0.09 ± 0.05	7.28 ± 1.95	0.83 ± 0.17
16 °C	t0	8 (4:4)	20.69 ± 2.14	138.14 ± 40.01	1.51 ± 0.12	1.99 ± 0.54	0.23 ± 0.02	0.10 ± 0.08	5.56 ± 1.69	0.38 ± 0.12
	t7	8 (6:2)	21.56 ± 2.31	152.29 ± 45.19	1.48 ± 0.20	2.25 ± 0.57	0.21 ± 0.06	0.07 ± 0.01	6.01 ± 2.82	0.47 ± 0.06
	t14	8 (7:1)	21.50 ± 2.49	153.30 ± 52.76	1.49 ± 0.11	2.21 ± 0.55	0.22 ± 0.08	-	6.72 ± 2.43	0.66 ± 0.14
	t21	8 (7:1)	22.63 ± 1.83	163.82 ± 33.24	1.40 ± 0.08	1.88 ± 0.73	0.25 ± 0.05	0.05	6.20 ± 2.81	0.85 ± 0.14

Table 2. Values of Pearson's correlation coefficient for bivariate correlations performed among oxidative-stress markers (LPO, GR, GPX and CAT), CYPs (EROD, BROD, MROD, BFCOD, CECOD and ECOD) and phase II metabolism markers (GST and UDPGT). Abbreviations for enzymatic markers can be found in the corresponding sections throughout the text. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; – non-significant result. n = 64.

	LPO (muscle)	LPO (liver)	GR	GPX	CAT	GST	EROD	BROD	MROD	BFCOD	CECOD	ECOD
LPO (muscle)												
LPO (liver)	–											
GR	–	-0.332**										
GPX	–	–	0.488***									
CAT	–	–	-0.294*	–								
GST	0.266*	0.416**	-0.473***	–	–							
EROD	–	–	0.279*	0.361**	–	–						
BROD	–	–	0.254*	–	–	–	0.526***					
MROD	–	–	0.273*	0.371**	–	-0.361**	0.871***	0.642***				
BFCOD	–	–	–	–	–	–	–	0.403**	–			
CECOD	–	–	–	0.263*	–	–	0.781***	0.547***	0.704***	–		
ECOD	-0.311*	–	–	0.329*	–	–	0.673***	0.562***	0.768***	–	0.698***	
UDPGT	–	–	–	–	0.347**	–	-0.315*	–	-0.287*	0.259*	–	-0.297*

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Table 3. Concentration of the chemical compounds fipronil and fipronil-sulfone determined in bile and of fipronil in muscle of European sea bass exposed to fipronil under two temperature regimes before exposure (t0) after 7 and 14 days of exposure (t7 and t14, respectively) and after a 7-day depuration period following exposure (t21). Each value corresponds to a pool of samples from 8 fish individuals. Quantification in bile at t7 was not possible due to sample limitations. LOD: limit of detection.

T °C	Time	Muscle	Bile	
		Fipronil (ng/g)	Fipronil (ng/ml)	Fipronil sulfone (ng/ml)
13 °C	t0	< LOD	< LOD	< LOD
	t7	88.4	–	–
	t14	64.6	4.08 ± 7.35	10.84 ± 9.02
	t21	< LOD	0.76 ± 0.47	15.17 ± 8.03
16 °C	t0	< LOD	< LOD	< LOD
	t7	42.9	–	–
	t14	57.7	4.70 ± 4.65	38.43 ± 20.16
	t21	< LOD	0.63 ± 0.62	34.58 ± 30.87

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Figure captions

Figure 1. Histograms displaying glucose and lactate levels in skin mucus (A and B, respectively) and plasma (C and D, respectively) of European sea bass exposed to fipronil under two temperature regimes before exposure (t0) after 7 and 14 days of exposure (t7 and t14, respectively) and after a 7-day depuration period following exposure (t21). Different letters show differences across temporal replicates (One-way ANOVA, $p < 0.05$). No differences between temperatures were detected.

Figure 2. Histograms displaying lipid peroxidation levels (LPO) (B) and activity levels of the enzymes catalase (CAT, A), glutathione-*S*-transferase (GST, C), Cytochrome P450-related BROD and MROD (D and E, respectively), NADPH Cytochrome C reductase (F), trypsin (G) and maltase (H) (the two latter in anterior intestinal region) in different tissues of European sea bass exposed to fipronil under two temperature regimes before exposure (t0) after 7 and 14 days of exposure (t7 and t14, respectively) and after a 7-day depuration period following exposure (t21). Different letters show differences across temporal replicates (One-way ANOVA, $p < 0.05$). No differences between temperatures were detected, except for NADPH Cyt C reductase.

Figure 3. Plots showing first and second components (A) and first and third components (B) of the principal components analysis (PCA) applied on biochemical data of European sea bass exposed to fipronil under two temperature regimes (filled symbols, 13 ºC; empty symbols, 16 ºC) before exposure (triangles, t0) after 7 and 14 days of exposure (squares, t7 and circles, t14) and after a 7-day depuration period following exposure (rhombus, t21). Fish data were grouped according to combination of temperature and time conditions. Vectors represent Pearson' correlations between each variable and the PCA axis. The outer circle represents a correlation = 1.

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A) **Glucose (plasma)**

B) **Lactate (plasma)**

C) **Glucose (skin mucus)**

D) **Lactate (skin mucus)**

□ 13 °C   ■ 16 °C

A) CAT

B) LPO (liver)

C) GST

D) BROD

E) MROD

F) NADPH Cyt C reductase

G) Trypsin (anterior)

H) Maltase (anterior)

□ 13 °C
■ 16 °C

A)

PCO2 (20.7% of total variation) vs PCO1 (35.4% of total variation)

Lactate/prot (mucus)
Osmolality
LPO (muscle)
Glucose (plasma)
Lactate (plasma)
LPO (liver)
Lysozyme
Glucose/prot (mucus)
CAT
Lactate (mucus)
Glucose (mucus)
EROD
CECOD
MROD
BROD
NADH Cyt C red.
GST
NADPH Cyt C red.
BFCOD
NADH Ferri-cyanide red.
GR
K+/Na+ ATPase
Glucose/lactate (mucus)
LDH
UDPGT
GPX

B)

PCO3 (16.5% of total variation) vs PCO1 (35.4% of total variation)

Glucose/lactate (mucus)
BFCOD
Glucose (mucus)
NADPH Cyt C red.
Glucose/prot (mucus)
Glucose (plasma)
BROD
K+/Na+ ATPase
LDH
Lactate/prot (mucus)
Lactate (mucus)
LPO (liver)
CECOD
Osmolality
MROD
Lactate (plasma)
GR
CAT
EROD
Lysozyme
NADH Ferricyanide red.
NADH Cyt C red.
GST
LPO (muscle)
UDPGT
GPX

A

QC2_10_PPB_FIPRONIL - FIPRONIL 1 (Quality C...RONIL_BILIS_MRM.wiff2), (sample Index: 54)
Area: 3.528e5, Height: 1.897e5, RT: 2.76 min

XIC — TOF-MS — MS/MS

Spectrum from 250818_FIPRONIL_BILIS_MRM.wif...NIL, Experiment 1, from 2.775 to 2.787 min

Spectrum from 250818_FIPRONIL_BILIS_MRM.wiff... to 2.883 min Precursor: 434.9 Da, CE: -20]

▼ Peak Details

Fragment m/z	Fragment Mass Error (ppm)	Retention Time (min)	Ion Ratio
329.960	-2.2	2.76	1.0000

▼ Formula Finder Results

Name	Formula	Score	m/z (Da)	Error (ppm)	Error MSMS (ppm)	Hit

▼ Library Search Results

Name	CAS#	Formula	MM (Da)	Fit	Rev. Fit	Purity	CE (eV)

B

QC3_10_PPB_FIPRONIL - Fipronil Sulfone 1 (Q...PRONIL_BILIS_MRM.wiff2), (sample Index: 76)
Area: 5.537e5, Height: 2.931e5, RT: 2.84 min

XIC — TOF-MS — MS/MS

Spectrum from 250818_FIPRONIL_BILIS_MRM.wif...NIL, Experiment 1, from 2.837 to 2.848 min

Spectrum from 250818_FIPRONIL_BILIS_MRM.wiff... to 2.967 min Precursor: 450.9 Da, CE: -20]

▼ Peak Details

Fragment m/z	Fragment Mass Error (ppm)	Retention Time (min)	Ion Ratio
414.951	-2.2	2.84	1.0000

▼ Formula Finder Results

Name	Formula	Score	m/z (Da)	Error (ppm)	Error MSMS (ppm)	Hit

▼ Library Search Results

Name	CAS#	Formula	MM (Da)	Fit	Rev. Fit	Purity	CE (eV)

C

QC3_10_PPB_FIPRONIL - FIPRONIL-DESULFINYL ...ONIL_BILIS_MRM.wiff2), (sample Index: 76)
Area: 4.833e5, Height: 2.984e5, RT: 2.73 min

XIC — TOF-MS — MS/MS

Spectrum from 250818_FIPRONIL_BILIS_MRM.wif...NIL, Experiment 1, from 2.731 to 2.742 min

Spectrum from 250818_FIPRONIL_BILIS_MRM.wiff... to 2.839 min Precursor: 387.0 Da, CE: -20]

▼ Peak Details

Fragment m/z	Fragment Mass Error (ppm)	Retention Time (min)	Ion Ratio
350.989	-5.2	2.73	1.0000

▼ Formula Finder Results

Name	Formula	Score	m/z (Da)	Error (ppm)	Error MSMS (ppm)	Hit

▼ Library Search Results

Name	CAS#	Formula	MM (Da)	Fit	Rev. Fit	Purity	CE (eV)

A

FIPRONIL_F21_13 - Fipronil Sulfone 1 (Unkno...PRONIL_BILIS_MRM.wiff2), (sample Index: 72)
Area: 4.561e3, Height: 2.577e3, RT: 2.85 min

XIC

Spectrum from 250818_FIPRONIL_BILIS_MRM.wif..._13, Experiment 1, from 2.831 to 2.842 min

TOF-MS

Spectrum from 250818_FIPRONIL_BILIS_MRM.wiff... to 2.973 min Precursor: 450.9 Da, CE: -20]

MS/MS

▼ Peak Details

Fragment m/z	Fragment Mass Error (ppm)	Retention Time (min)	Ion Ratio
414.951	1.1	2.85	1.0000

▼ Formula Finder Results

Name	Formula	Score	m/z (Da)	Error (ppm)	Error MSMS (ppm)	Hit (

▼ Library Search Results

Name	CAS#	Formula	MM (Da)	Fit	Rev. Fit	Purity	CE (eV)

B

FIPRONIL_F21_13 - FIPRONIL-DESULFINYL 1 (U...RONIL_BILIS_MRM.wiff2), (sample Index: 72)
Area: 2.436e4, Height: 1.915e4, RT: 2.74 min

XIC

Spectrum from 250818_FIPRONIL_BILIS_MRM.wif..._13, Experiment 1, from 2.742 to 2.753 min

TOF-MS

Spectrum from 250818_FIPRONIL_BILIS_MRM.wiff... to 2.823 min Precursor: 387.0 Da, CE: -20]

MS/MS

▼ Peak Details

Fragment m/z	Fragment Mass Error (ppm)	Retention Time (min)	Ion Ratio
350.989	-2.8	2.74	1.0000

▼ Formula Finder Results

Name	Formula	Score	m/z (Da)	Error (ppm)	Error MSMS (ppm)	Hit (

▼ Library Search Results

Name	CAS#	Formula	MM (Da)	Fit	Rev. Fit	Purity	CE (eV)

Electronic supplementary material (ESM)

Materials and Methods

Chemical analyses on bile

Fipronil reference standards (fipronil, fipronil sulfone, fipronil-desulfinyl, and fipronil-(pyrazole-$^{13}C_3$, cyano-^{13}C)) were high purity (≈90 %) and were obtained from Sigma Aldrich (St. Luis, MO, U.S). LC-MS grade acetonitrile (ACN) (≥99.9 %), methanol (MeOH) (≥99.9 %) and water were purchased from Merck (Darmstadt, Germany). Formic acid (≥96 %, ACS reagent) and ammonium acetate were supplied by Sigma-Aldrich. Polypropylene 96-well plates, 700 µL were furnished by Waters Corporation (Milford, MA, US). Fipronil, fipronil sulfone and fipronil-desulfinyl stock standard solutions (1000 mg/L) were prepared in, and the working standard solutions required for quantification were prepared from stock solution by dilution with ACN. All standard solutions were stored at −20 °C before use. Fipronil-(pyrazole-$^{13}C_3$, cyano-^{13}C) stock solution (1000 mg/L) used as internal standard (IS) was prepared in ACN and stored at −20 °C.

The extraction methodology was as follows. Briefly, the 2-mL Eppendorf vials containing the glands were slowly defrosted in an ice bath for 30 minutes. Then, glands were incised with a syringe needle to allow bile to leak, the bag was removed and the vials were vortexed for 30 seconds. Sixty microliters of bile content were added to 675 µL of 0.1 N HCl and vortexed for 30 seconds. Then, 200 µL of cold ACN and 25 µL of IS solution (1000 µg/L) were added (final volume 960 µL) and the vial was vortexed for 30 seconds more. Subsequently, the vials were centrifuged for 10 minutes at 10,000 rpm and 4 °C using a 5810 R centrifuge (Eppendorf AG, Hamburg, Germany). About 800 µL of the supernatants were transferred to a polypropylene 96-deepwell plate for the following analysis by ultra-high-performance liquid chromatography (UPLC)-high

resolution multiple reaction monitoring (HR-MRM) for quantification of target compounds. To evaluate the method extraction, a blank sample (control, in triplicate) fortified at 10 ng/mL was run in parallel to the set of samples (Supplementary material, Figure S1).

All analyses were performed using a SCIEX ExionLC™ AD system coupled to a hybrid SCIEX X500R QTOF system (Sciex, Redwood City, CA, U.S.) equipped with a Turbo V™ source and Electrospray Ionization (ESI). Fipronil and its metabolites were separated on a reverse phase Hibar® HR Purospher® STAR RP-C18 column (50 mm × 2.1 mm i.d., 2 μm particle size, Merck, Darmstadt, Germany), maintained at 40 °C in the column oven. The mobile phases consisted of 5 mM ammonium formate in water (A) and 5 mM ammonium formate buffer and 0.05 % formic acid MeOH (B) at a flow rate of 0.6 mL/min. The gradient elution program was as follows: 0.3–1.10 min 5–40 % B, 1.10–2.20 min 40–80 % B, 2.20–3.60 min 80–87 % B, 3.60–4.00 min 87–95 % B, 4.00–5.00 min 5 % B. The injection volume was 5 μL and the auto-sampler temperature was maintained at 8 °C. The QTOF system was operated in ESI negative with multiple reaction monitoring scan mode in high resolution (MRM-HR).

High resolution data were acquired using a multiple reaction monitoring (MRM[HR]) workflow consisting of a TOF-MS survey (100–850 Da for 100 ms of Accumulation time (AT); Declustering Potential (DP) was set to –80 V and a Collision Energy (CE) to –10 V), and MRM[HR] scanning mode was used for accurate quantification of product ion transitions. The Guided MRM[HR] tool from SCIEX was used for the optimization of high resolution transitions (Fig. S2). The source conditions for the system were optimized as follow. Ion Spray Voltage was set to –4500 V; Source temperature and nitrogen gas flows (Atomizing gas, GS1 and Auxiliary gas, GS2) were set to 550 °C and 50 psi,

respectively. Curtain gas was set to 30 psi, Declustering Potential (DP) was set to −80 V.

Qualitative and quantitative analyses were performed using SCIEX OS™ Software version 1.4 (Sciex, Redwood City, CA, U.S.). According to SANTE European Commission guideline for pesticides (SANTE/11813/2017), two ions with mass accuracy equal or mass difference lower than 5 ppm are necessary for confirming a positive finding for the identification in HR-QToF-MS analysis. In the present study, two high resolution ions were used for each compound, the most abundant product ion for the quantification and the precursor ion for the confirmation. Any drift in the mass accuracy of the SCIEX Q-TOF was automatically corrected and maintained throughout batch acquisition by infusion of a cluster of trifluoroacetic acid (5(TFA-Na)+TFA-, m/z 792.85963) for negative mode. Calibration was running every 5 samples during the batch acquisition making use of the Calibrant Delivery System (CDS).

Chemical analyses on muscle

Ethyl acetate was the solvent used for gas chromatography-ECD and FID from Merck (Darmstadt, Germany). QuEChERS Final Polish and QuEChERS dSPE EMR-Lipid were from Agilent (Santa Clara, CA, USA). Samples were freeze dried on a Telstar LyoAlfa 6 freeze dryer during 24 h at −80 ºC and 0.1 mbar.

A standard stock solution of commercial Regent®800WG (80% fipronil) at 1000 µg/mL in ethyl acetate was prepared, step-wise diluted to 100 ng/mL and used to spike QuEChERS to assess recovery throughout the analytical procedure. Six QuEChERS Final Polish were spiked with 0, 50, 100, 150, 200 and 250 µL of the 100 ng/mL fipronil standard solution, respectively. A 6 points calibration curve ranging from 100 to 1000 ng/mL was prepared from the 1 µg/mL fipronil standard solution with ethyl

acetate as a solvent. Standard solutions of 100, 200, 300, 400, 500 and 1000 ng/mL were used to calibrate the Gas Chromatography-Mass Spectrometry (GC/MS) instrument before the samples and spiked tubes analysis. Sample preparation was based on the QuEChERS method with ethyl acetate. 0.5 g of freeze dried fish muscle sample were weighed in a QuEChERS Final Polish tube and 10 mL ethyl acetate were added. The tube was hand-shaken until total solid suspension. QuEChERS Final Polish tubes were then vortexed for 1 minute and centrifuged for 10 minutes at 5,000 rpm, tap covered. 6 mL supernatant were transferred to a QuEChERS dSPE EMR-Lipid tube, vortexed for 2 minutes and centrifuged 5 minutes at 5,000 rpm at room temperature. 4 mL supernatant were evaporated to dryness under a gentle stream of nitrogen. Extracts were solved in 50 μL ethyl acetate, 2 μL to be injected in the GC/MS instrument. A GC/MS equipment QP 2010 from Shimadzu (Kyoto, Japan) was used to instrumentally determine fipronil in muscle samples. Analyser was used in electronic ionization mode. Column used was a SPB-1 Supelco (Bellefonte, PA, USA) 30 m long, 0.25 mm d.i. and 0.25 μm film thickness. Injector temperature was 260 ºC. The GC oven temperature program was as follows: 60 ºC held for 1 min, ramped at 20 ºC/min to 160 ºC held for 1 min, then ramped at 3 ºC/min to 200 ºC held for 2 min, followed by 4 ºC/min to 250 ºC and held for 4 min. MSD transfer line was at 250 ºC, and ion source was set at 200 ºC. Electron impact energy was −70 eV, solvent delay was 5 min and detector gain was 2.0 kV. Carrier gas was helium at a flow of 1.00 mL/min. Acquisition mode was SIM (Selected ion monitoring) for m/z 367, 369, 213, 255 (fipronil most abundant ions in the scan mass spectrum). For method evaluation, a blank and 5 standard spiked tubes were run in parallel to the set of samples.

Data analyses

Prior to statistical treatment, Kolmogorov-Smirnov test was used for testing normality and Levene´s test for checking homoscedasticity of all variables. The presence of outliers was evaluated and some variables were log- or square-root transformed to comply with normality and homoscedasticity requirements. Relationships among biological variables (SL, BW, HSI, GSI, CF, visceral fat and sex) and between them and biochemical variables were tested by Pearson' or Spearman' rank correlations (in the case of continuous variables) and by Student's t-tests or Mann-Whitney U-tests (for sex-related differences).

Differences in the level of activity of digestive enzymes between anterior and posterior parts of the digestive tract were tested for each experimental temperature using the Wilcoxon pairwise test with repeated measurements using PAST 3.

Possible effects of treatment duration (four categories: t0, t7, t14 and t21) and temperature (two categories: 13 ºC and 16 ºC) on fish biological and biochemical variables were tested by general linear models (GLMs) or generalized linear models (GZMs). Fish biological variables that had previously displayed significant correlations with biomarkers were incorporated as covariates in GLMs/GZMs to test possible interactions with the two factors assessed (i.e. treatment duration and temperature). Afterwards, Student's t-test/Mann-Whitney U-test and one-way ANOVA/Kruskal-Wallis tests were performed to determine differences between/among categories of factors temperature and time, respectively, when a significant effect was detected in previous GLMs/GZMs.

Pearson' or Spearman' rank correlations were used to explore relationships among activities of CYPs in liver microsomal fraction, among antioxidant enzymes in liver cytosolic fraction and between both. Associations among metabolites quantified within plasma and mucus, and between metabolites of both matrices were assessed in the same

way. Significant associations among digestive enzymes activities were assessed by means of a Spearman' rank correlations in anterior and posterior portions of digestive tract.

A permutation multivariate analysis (PERMANOVA) was carried out using individual fish as replicate samples in order to assess a global biochemical response to the factors temperature and treatment duration (i.e. exposure to fipronil). Permutation p-values were obtained under unrestricted permutation of raw data (9999 permutations). Finally, a principal component analysis (PCA) was performed and plotted in two dimensions. Pearson's correlation between each variable and PCA axes was calculated. The two multivariate analyses were applied on a Euclidean distance-based matrix derived from normalized data after square-root transformation. For PCA, data were grouped according to combination of temperature with treatment durations. All biomarkers included in the present work were considered in these analyses, with the exception of digestive enzymes, plasma ammonia content and ECOD activity, for which an excessive number of missing values occurred.

Student t-test, Mann-Whitney U-test, Kruskal-Wallis test, one-way ANOVA, GLM, GZM and correlation tests were run in IBM SPSS (Statistics for Windows, Version 20.0) or Statistica 6 (StatSoft®, Richmond, USA). Wilcoxon pairwise tests with repeated measurements were performed using PAST 3. Multivariate analyses were carried out using PRIMER PERMANOVA+ v6 (Anderson et al., 2008).

Results

Chemical analyses on bile

Levels of fipronil-desulfinyl quanified in bile were below the limit of quantitation (LOQ) and thus not reported. MS/MS conditions of the target compounds were

optimized in negative HR ESI mode. The optimal MRM-HR parameters including precursor ions, product ions, DP, and CE are shown in Table S7. Linearity was determined using the peak areas of the product ions obtained from the MRM-HR acquisition. Results are provided in Table S8. The standard calibration curves consisted of 9 concentration levels (0.05-200/ng mL) and were set up by plotting the target analyte concentrations against the peak areas. However, the TOF detector responses were linear from 0.05 to 10 ng/mL ($R^2 < 0.998$). A recovery experiment was performed to determine the accuracy and precision of the extraction method fortifying 3 blank samples at 10 ng/mL (Fig. S1). The average recoveries of fipronil and its metabolites ranged from 93.3% to 109.3% with a relative standard deviation (RSD) of 1.7–3.8% (n = 3) (Table S8). The limits of detection (LODs) were calculated at 3 times the signal to noise ratio generated from all the samples, and the LOQs were taken as the minimum concentration of a compound that can be measured by the instrument; both are reported in Table S8.

Chemical analysis on muscle

Regarding method evaluation, ion *m/z* 367 was the base peak in fipronil mass spectrum, but an interference with this *m/z* ion was detected in blank samples. Recoveries from the spiked tubes calculated using *m/z* 367 as quantitation ion were inconsistent and over 100 % in some cases. In order to disregard interferences, quantitation ion was changed to *m/z* 369. In this case, recoveries were low but consistent: mean 29.7 %, standard deviation 2.7 % (n = 4). An outlier result was disregarded. Sample concentrations were then corrected by this recovery mean result.

References

Anderson, M.J., Gorley, R.N., Clarke, K.R., 2008. PERMANOVA+ for PRIMER: guide to software and statistical methods. PRIMER-E, Plymouth, UK.

SANTE/11813/2017: Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed. rev.0, 21–22 November 2017, European Commission, Brussels, 42 pp.

Tables

Table S1. Mean ± standard error of the mean for metabolites quantified in plasma and skin mucus, and plasmatic osmolality, of seabass exposed to Fipronil under two temperature regimes before exposure (t0), after 7 and 14 days of exposure (t7 and t14, respectively) and after a 7-day depuration period following exposure (t21). Biomarker units can be found in the corresponding sections throughout the text.

Tissue	Biomarker	13 ºC				16 ºC			
		t0	t7	t14	t21	t0	t7	t14	t21
Plasma	Glucose	11.04 ± 1.42	19.04 ± 2.60	15.23 ± 1.91	21.23 ± 2.22	11.86 ± 1.46	19.19 ± 1.99	15.66 ± 0.93	15.58 ± 1.32
	Lactate	5.27 ± 0.55	6.36 ± 1.11	6.50 ± 0.45	7.81 ± 0.88	5.83 ± 0.41	7.93 ± 0.71	6.60 ± 0.44	7.55 ± 0.54
	Osmolality	370.50 ± 4.69	390.86 ± 8.03	383.29 ± 4.92	392.63 ± 3.98	374.57 ± 5.61	397.29 ± 4.92	395.00 ± 7.15	400.50 ± 9.12
Mucus	Glucose	1.17 ± 0.22	1.10 ± 0.14	0.88 ± 0.10	0.57 ± 0.07	1.09 ± 0.09	1.10 ± 0.15	1.24 ± 0.11	0.60 ± 0.09
	Lactate	0.74 ± 0.13	0.71 ± 0.16	0.76 ± 0.11	0.42 ± 0.08	0.64 ± 0.09	1.08 ± 0.12	1.11 ± 0.14	0.49 ± 0.09
	Glucose/protein	6.15 ± 1.67	6.39 ± 0.98	3.31 ± 0.47	5.99 ± 1.42	4.02 ± 0.61	4.97 ± 0.75	4.64 ± 0.64	4.11 ± 0.54
	Lactate/protein	4.00 ± 0.92	3.57 ± 0.65	2.70 ± 0.36	3.30 ± 0.56	2.28 ± 0.24	4.59 ± 0.37	3.94 ± 0.50	3.10 ± 0.50
	Glucose/lactate	1.61 ± 0.25	1.65 ± 0.21	1.37 ± 0.10	1.95 ± 0.29	1.67 ± 0.16	1.06 ± 0.12	1.22 ± 0.15	1.17 ± 0.11

Table S2. Values of statistical parameters obtained when testing relationships between biomarkers and fish biological variables (SL: standard length, TW: total weigth, CF: condition factor, HSI: hepatosomatic index and GSI: gonadosomatic index). Pearson's or Spearman correlation coefficients are provided for bivariate correlations (in the case of continuous variables) and t statistic for Student's t-test (for differences between sexes). Abbreviations an units for enzymatic markers can be found in the corresponding sections throughout the text. * $p<0.05$; **$p<0.01$; *** $p<0.001$; – non-significant result. n = 64 (except for digestive biomarkers, for which n = 48).

Tissue	Biomarker	SL	TW	CF	HSI	GSI	Visceral fat weight	Sex
Muscle	LPO	–	–	–	–	–	–	–
Liver - S9	LDH	–	–	–	–	–	–	–
	LPO	0.286*	–	–	–	–	–	–
Liver - cytosol	GR	-0.336*	-0.494***	-0.299*	–	–	-0.333**	–
	t-GPX	–	–	–	–	–	–	–
	CAT	–	–	–	–	–	–	–
	GST	0.308*	0.423**	0.305*	–	–	0.320*	2.707**
Liver -microsomes	EROD	–	–	–	-0.484***	–	-0.273*	–
	BROD	–	–	–	-0.324**	–	–	–
	MROD	–	–	–	-0.587***	–	-0.293*	–
	BFCOD	–	–	–	–	–	–	–
	CECOD	–	–	–	-0.262*	–	–	–
	ECOD	–	–	-0.292*	-0.651***	–	–	–
	UDPGT	–	–	0.391**	0.440***	–	0.285*	–
	NADPH-Cyt C reductase	–	–	–	–	–	0.287*	–
	NADH-Cyt C reductase	–	–	–	–	-0.257*	–	–
	NADH-Ferricyanide reductase	–	–	–	–	–	–	–
Kidney	Na$^+$/K$^+$-ATPase	–	–	–	–	–	–	–
Plasma	Glucose	0.315*	0.430**	0.357**	0.413**	–	0.375**	–
	Lactate	–	0.320*	0.511***	0.406**	–	0.300*	–
	Osmolality	–	–	–	–	–	–	–
	Ammonia	–	–	–	0.312*	–	0.336*	–
	Lysozyme	0.287*	0.438**	0.270*	0.297*	0.290*	0.434**	–
Skin mucus	Glucose	–	–	0.268*	–	–	–	–
	Lactate	–	–	0.358**	–	–	0.305*	–
	Glucose/protein	0.281*	0.255*	–	–	–	–	–
	Lactate/protein	–	0.293*	–	–	–	–	–
	Glucose/lactate	–	–	–	–	–	–	–
Digestive tract - anterior	Trypsin	–	–	–	–	–	–	–
	Chymotrypsin	–	–	–	–	–	-0.323*	–
	Lipase	–	–	–	–	–	–	–
	Esterases	–	–	0.342*	0.427**	–	–	–
	Amylase	–	–	–	–	–	–	–
	Alkaline phosphatase	–	–	-0.453**	-0.496**	–	–	–
	Aminopeptidase	–	–	–	0.318*	–	–	–
	Maltase	–	–	–	–	–	–	–
Digestive tract - posterior	Trypsin	–	–	–	–	–	–	–
	Chymotrypsin	–	–	–	0.336*	–	–	–
	Lipase	0.304*	–	–	–	–	–	–
	Esterases	–	–	–	–	–	–	–
	Amylase	–	–	–	–	–	–	–
	Alkaline phosphatase	–	-0.438**	-0.313*	-0.475**	–	-0.394**	–
	Aminopeptidase	–	–	–	–	–	–	–
	Maltase	–	–	–	–	–	–	–

Table S3. Mean ± standard error of the mean for enzymatic markers quantified in muscle, liver, kidney and plasma of seabass exposed to Fipronil under two temperature regimes before exposure (t0), after 7 and 14 days of exposure (t7 and t14, respectively) and after a 7-day depuration period following exposure (t21). Abbreviations and units for biomarkers can be found in the corresponding sections throughout the text.

Tissue	Biomarker	13 ºC				16 ºC			
		t0	t7	t14	t21	t0	t7	t14	t21
Plasma	Lysozyme	7.14 ± 0.38	11.60 ± 1.25	8.11 ± 0.70	10.73 ± 1.02	6.24 ± 1.45	9.03 ± 2.78	12.36 ± 0.50	13.24 ± 2.53
Muscle	LPO	2.99 ± 0.74	3.03 ± 0.46	5.57 ± 1.37	4.12 ± 0.72	3.14 ± 1.06	6.21 ± 1.09	5.30 ± 0.70	5.41 ± 1.88
Liver (S9)	LDH	15.11 ± 0.90	14.85 ± 1.11	16.25 ± 1.17	16.23 ± 1.00	15.48 ± 1.48	14.58 ± 1.42	15.61 ± 1.37	15.49 ± 1.38
	LPO	10.10 ± 1.74	15.81 ± 1.72	14.12 ± 1.57	16.82 ± 2.18	10.15 ± 1.45	13.69 ± 1.21	13.02 ± 1.62	17.35 ± 1.96
Liver (cytosol)	GR	7.48 ± 1.02	6.03 ± 0.86	6.85 ± 0.68	7.98 ± 0.84	7.39 ± 0.63	7.20 ± 0.94	5.56 ± 1.05	7.84 ± 1.17
	t-GPX	7.11 ± 0.37	6.60 ± 0.40	6.70 ± 0.33	7.16 ± 0.54	8.12 ± 0.40	6.64 ± 0.38	7.13 ± 0.52	7.97 ± 0.31
	CAT	103.68 ± 12.47	90.70 ± 8.68	92.85 ± 11.35	63.84 ± 10.27	96.70 ± 7.19	108.92 ± 8.75	111.12 ± 6.28	76.88 ± 11.33
	GST	36.94 ± 1.73	39.49 ± 1.51	42.83 ± 3.52	43.26 ± 2.71	39.44 ± 2.62	36.45 ± 2.43	48.27 ± 2.52	51.05 ± 5.80
Liver (microsomes)	EROD	22.04 ± 2.09	18.75 ± 2.98	15.48 ± 1.38	15.33 ± 1.31	25.35 ± 3.86	24.36 ± 4.37	17.01 ± 3.23	18.14 ± 2.56
	BROD	0.69 ± 0.06	0.66 ± 0.07	0.54 ± 0.05	0.55 ± 0.07	0.76 ± 0.11	0.63 ± 0.07	0.49 ± 0.08	0.47 ± 0.07
	MROD	4.70 ± 0.32	3.74 ± 0.51	2.80 ± 0.34	2.34 ± 0.18	5.00 ± 0.71	4.17 ± 0.64	2.91 ± 0.60	3.40 ± 0.78
	BFCOD	62.91 ± 5.23	88.69 ± 13.95	94.29 ± 11.36	98.97 ± 22.34	87.23 ± 19.95	65.81 ± 10.38	68.65 ± 12.07	64.05 ± 9.80
	CECOD	31.06 ± 5.11	30.65 ± 4.68	19.14 ± 1.63	21.66 ± 2.09	33.45 ± 5.34	29.98 ± 3.73	23.44 ± 4.39	24.80 ± 2.31
	ECOD	9.42 ± 1.47	8.51 ± 1.66	3.99 ± 1.05	3.35 ± 0.66	10.03 ± 3.17	6.34 ± 1.40	6.53 ± 3.52	7.18 ± 1.97
	UDPGT	0.78 ± 0.09	0.76 ± 0.07	0.77 ± 0.14	0.78 ± 0.08	0.81 ± 0.13	0.75 ± 0.09	0.77 ± 0.08	0.82 ± 0.08
	NADPH Cyt C reductase	19.42 ± 0.98	19.06 ± 1.08	22.14 ± 1.57	22.98 ± 2.24	18.04 ± 1.49	17.07 ± 1.22	18.99 ± 1.11	18.94 ± 1.44
	NADH Cyt C reductase	31.94 ± 2.33	23.58 ± 4.11	22.79 ± 3.36	23.12 ± 3.84	31.97 ± 5.34	28.69 ± 3.80	26.15 ± 4.86	29.02 ± 3.60
	NADH Ferricyanide reductase	1079 ± 93	1145 ± 111	1167 ± 103	1095 ± 66	1184 ± 107	1006 ± 95	1208 ± 108	1184 ± 75
Kidney	Na^+/K^+-ATPase	1.94 ± 0.51	1.17 ± 0.14	1.39 ± 0.27	1.40 ± 0.13	1.10 ± 0.17	0.91 ± 0.11	1.09 ± 0.22	1.35 ± 0.17

Table S4. Mean ± standard error of the mean for enzymatic markers quantified in anterior and posterior parts of the digestive tract of seabass exposed to Fipronil under two temperature regimes before exposure (t0), after 14 days of exposure (t14) and after a 7-day depuration period following exposure (t21). Units for biomarkers can be found in the corresponding sections throughout the text.

Tissue	Fraction		13 °C			16 °C		
			t0	t14	t21	t0	t14	t21
Digestive tract anterior	Pancreatic	Trypsin	51.00 ± 10.56	60.80 ± 2.25	48.80 ± 1.25	49.90 ± 7.52	61.20 ± 2.63	45.60 ± 3.07
		Chymotrypsin	104.70 ± 33.26	94.70 ± 32.25	80.50 ± 10.13	95.40 ± 33.75	69.00 ± 24.63	61.60 ± 15.87
		Lipase	42.70 ± 11.33	23.50 ± 2.48	21.80 ± 3.23	27.30 ± 3.52	34.10 ± 4.57	21.50 ± 3.73
		Alpha-amylase	7.70 ± 1.33	4.40 ± 0.92	4.50 ± 1.54	5.50 ± 0.41	5.30 ± 0.92	5.70 ± 1.26
		Non-specific esterases	523.70 ± 55.64	517.70 ± 77.30	457.90 ± 34.76	541.50 ± 32.53	564.40 ± 35.19	415.60 ± 38.22
	Brush border	Alkaline-phosphatase	918.00 ± 309.79	966.60 ± 233.57	1,023.40 ± 147.92	1,026.40 ± 163.29	742.40 ± 116.31	1,177.40 ± 284.87
		Aminopeptidase	148.10 ± 24.04	218.80 ± 33.60	220.50 ± 22.80	177.80 ± 25.03	117.30 ± 22.32	149.20 ± 22.70
		Maltase	42.90 ± 8.63	250.10 ± 50.02	242.30 ± 24.02	43.50 ± 3.30	127.10 ± 21.62	214.00 ± 39.23
Digestive tract posterior	Pancreatic	Trypsin	57.10 ± 12.98	47.70 ± 2.55	47.80 ± 1.78	64.40 ± 10.03	52.10 ± 3.38	45.70 ± 3.22
		Chymotrypsin	117.30 ± 39.39	231.40 ± 84.12	176.10 ± 59.25	340.30 ± 142.84	180.20 ± 85.15	332.30 ± 145.94
		Lipase	30.00 ± 5.69	30.80 ± 3.94	30.40 ± 6.06	27.30 ± 8.46	28.60 ± 3.71	24.90 ± 3.82
		Alpha-amylase	3.10 ± 0.44	4.90 ± 0.79	9.70 ± 2.48	3.40 ± 0.40	5.80 ± 1.15	6.20 ± 1.52
		Non-specific esterases	587.70 ± 56.37	477.20 ± 43.85	519.70 ± 27.89	481.00 ± 12.44	553.70 ± 58.99	447.40 ± 43.69
	Brush border	Alkaline-phosphatase	626.70 ± 193.56	826.20 ± 175.18	749.70 ± 97.03	776.90 ± 95.46	540.80 ± 109.19	745.00 ± 176.76
		Aminopeptidase	97.20 ± 6.81	159.30 ± 21.09	178.90 ± 27.34	154.20 ± 19.76	93.50 ± 11.08	104.70 ± 15.78
		Maltase	26.20 ± 2.92	158.20 ± 23.95	150.50 ± 25.26	32.80 ± 2.44	93.60 ± 11.88	116.30 ± 17.48

Table S5. Values of Pearson's coefficient for bivariate correlations performed among the activities of digestive enzymes in the anterior part of the digestive tract. * p<0.05; **p<0.01; *** p<0.001. – non-significant result.

	Trypsin	Chymotrypsin	Lipase	Non-specific esterases	Alpha-amylase	Alkaline phosphatase	Aminopeptidase	Maltase
Trypsin								
Chymotrypsin	–							
Lipase	–	–						
Non-specific esterases	–	–	0.537***					
Alpha-amylase	–	–	0.411**	0.326*				
Alkaline phosphatase	–	–	–	-0.362*	–			
Aminopeptidase	–	–	–	–	–	0.343*		
Maltase	–	–	–	–	-0,420**	0.482**	0.516***	

Table S6. Values of Pearson's coefficient for bivariate correlations performed among the activities of digestive enzymes in the posterior part of the digestive tract. * p<0.05; **p<0.01; *** p<0.001. – non-significant result.

	Trypsin	Chymotrypsin	Lipase	Non-specific esterases	Alpha-amylase	Alkaline phosphatase	Aminopeptidase	Maltase
Trypsin								
Chymotrypsin	–							
Lipase	–	–						
Non-specific esterases	–	–	0.550***					
Alpha-amylase	–	0.351*	–	–				
Alkaline phosphatase	–	-0.331*	–	–	–			
Aminopeptidase	–	–	–	–	–	0.593***		
Maltase	–	–	–	–	–	0.504***	0.573***	

Table S7. Parameters for analysis of fipronil and its metabolites in bile: exact mass, transitions and retention time obtained by UPLC-HR-QToF-MS.

Compound ID	Precursor ion (m/z)	Fragment ion (m/z)	Retention time (min)	Accumulation time (sec)	Declustering potential (V)	Collision energy (V)
Fipronil	434.9309	329.96	2.76	0.05	-25	-20
Fipronil Sulfone	450.9258	414.9508	2.84	0.05	-25	-20
Fipronil-desulfinyl	386.9639	350.9889	2.72	0.05	-100	-20
Fipronil-(13C3, cyano-13C)	438.9317	333.9745	2.75	0.05	-25	-20

Table S8. Linear regression parameters of the calibration curve of fipronil and its metabolites in bile.

Compound	Linearity range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	R^2	Accuracy (%)
Fipronil	0.05-10	0.01	0.05	0.99963	109.3 (2.4)
Fip. Sulfone	0.05-10	0.01	0.05	0.99985	93.3 (1.7)
Fip. Desulfynil	0.05-10	0.01	0.05	0.99844	93.8 (3.8)

Figure captions

Figure S1. Extracted Ion Chromatograms (XIC), TOF/MS and MS/MS spectra of fipronil (A) and its metabolites, fipronil sulfone (B) and fipronil-desulfinyl (C) in fortified samples spiked at 10 ng/ml.

Figure S2. Extracted Ion Chromatograms (XIC), TOF/MS and MS/MS spectra of fipronil metabolites in Sample #13 after 21 days. A, fipronil sulfone and B, fipronil-desulfinyl.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Since this manuscript considers many disciplines all contributed to a particular topic:

I. Sanahuja: mucus and plasma biomarkers
M. Solovyevd and E. Gisbert: digestive enzymes
N. Montemurro: chemical analysis
A. Torreblanca: osmoregulatory parameters

S. Dallares, P. Dourado, M. Blazquez and M. Sole: designed and conducted the laboratory experiment, analysed the remaining parameters, coordinated and wrote the manuscript as well as the final English version of the revised manuscript.