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3 **1 Multibiomarker approach to fipronil exposure in the fish *Dicentrarchus labrax***
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5 **2 under two temperature regimes**
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62 **Abstract**
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64 Fipronil is a phenylpyrazole insecticide widely used to control pests in agriculture even
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67 though evidence of harmful side effects in non-target species has been reported. A
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70 comprehensive study on the effects of dietary administration of Regent®800WG (80%
71 fipronil) in European sea bass juveniles was carried out under two temperature regimes:
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73 a) natural conditions, and b) 3 °C above the natural temperature (an increase predicted
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75 for the NW Mediterranean by the end of this century). Fipronil was added to the fish
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77 food (10 mg fipronil /Kg feed) and the effects were studied at several time points
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79 including right before administration, 7 and 14 days after daily fipronil feed and one-
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81 week after the insecticide withdrawal from the diet (depuration period). A wide array of
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83 physiological and metabolic biomarkers including feeding rate, general condition
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85 indices, plasma and epidermal mucus metabolites, immune response, osmoregulation,
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87 detoxification and oxidative-stress markers and digestive enzymes were assessed.
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90 General linear models and principal component analyses indicated that regardless of
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92 water temperature, fipronil resulted in a significant alteration of several of the above
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94 listed biomarkers. Among them, glucose and lactate levels increased in plasma and
95
96 decreased in epidermal mucus as indicators of a stress response. Similarly, a depletion
97
98 in catalase activity and higher lipid peroxidation in liver of fipronil-exposed fish were
99
100 also indicative of an oxidative-stress condition. Fipronil induced a time dependent
101
102 inhibition of Cytochrome P450-related activities and an inhibition of phase II
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104 glutathione-S-transferase. Moreover, fipronil administration was able to reduce the
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106 hypo-osmoregulatory capability as shown by the increase of plasmatic osmolality and
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108 altered several digestive enzymes including trypsin, lipase, alpha amylase and maltase.
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111 Finally, analyses in bile and muscle confirmed the rapid clearance of fipronil but the
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113 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
59 change.

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180 **77 1. Introduction**
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182 78 The phenylpyrazole fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-
183 79 (trifluoromethylsulfonyl)pyrazole-3-carbonitrile) has been classified as moderately
184 80 hazardous (Class II) by the World Health Organisation (WHO, 2009). It is one of the
185 81 most used broad-spectrum insecticides in crops worldwide, being even effective against
186 82 pests resistant to pyrethroids, organophosphates and carbamate insecticides (Simon-
187 83 Delso et al., 2015). In Europe, fipronil is mainly used in crops of maize, rice and in
188 84 sunflower seed treatment. However, its use in agriculture was severely restricted by the
189 85 European Union in 2013 (Comission Implementing Regulation (EU) N° 781/2013) due
190 86 to its high acute toxicity for honeybees (European Food Safety Authority EFSA, 2013).
191 87 There is strong evidence that soils, aquatic systems and plants in agricultural
192 88 environments and their neighbouring areas are contaminated with fipronil and other
193 89 fipronil-related substances (US Environmental Protection Agency, 1996; Bonmatin et
194 90 al., 2015). Nevertheless, Spain, the largest fipronil end-user on sunflower crops in
195 91 Europe, is reluctant to adhere to the European directive alluding to the existence few on-
196 92 site studies evidencing its toxicity.
197 93 Fipronil and its main metabolites are toxic to non-target aquatic species (Schlenk et al.,
198 94 2001; Stefani Margarido et al., 2013; Gripp et al., 2017). This compound exerts its
199 95 insecticidal activity by binding to the gamma-aminobutyric acid (GABA) receptors and
200 96 acting as a non-competitive blocker of GABA-gated chloride channels in the central
201 97 nervous system, inducing neuronal hyperexcitation, paralysis and death (Simon-Delso et
202 98 al., 2015; Huang et al., 2019). Although fipronil is generally more toxic to invertebrates
203 99 than to vertebrates, due to differential affinity towards target receptors, a recent study on
204 100 bighead carp (*Hypophthalmichthys nobilis*) showed that the affinity of this chemical to
205 101 fish GABA receptors is similar to that found in insects, suggesting that it could also be

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238
239 102 highly toxic to fish (Zhang et al., 2018). Besides this, its main degradation products,
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241 103 which include fipronil-sulfone, fipronil-sulfide and fipronil-desulfinyl, are less specific
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243 104 than the parent compound, display higher insecticidal activity and also account for
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245 105 toxicity in vertebrates (Hainzl et al., 1998; Zhao et al., 2005; Lu et al., 2010; Gupta,
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247 106 2014; Gripp et al., 2017).

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250 107 Human activities, including the input of pesticides into the environment, have been
251
252 108 considered as the main cause for the present world climate change scenario (CC)
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254 109 (Hansen et al., 2006). Current consensus alerts that significant temperature increases,
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256 110 acidification and greater salinity fluctuations of marine water bodies will occur around
257
258 111 the globe in the upcoming decades (IPCC, 2014). These changes can exert a direct
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260 112 impact on the physiology of marine poikilotherms (Makrinos and Bowden, 2016;
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262 113 Boltana et al., 2017; Navarro et al., 2019). On the other hand, indirect effects caused by
263
264 114 CC in marine fish are still poorly known, especially those related to their potential
265
266 115 interaction with foreign chemicals (Schiedek et al., 2007; Hooper et al., 2013). For
267
268 116 instance, changes in physical conditions and chemical exposure can act synergistically
269
270 117 magnifying the consequences of such exposures in aquatic organisms, since the former
271
272 118 can imply changes in the availability and action of chemicals (Sokolova and Lannig,
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274 119 2008; Jacquin et al., 2019). The Mediterranean region is especially sensitive to the
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276 120 alterations induced by CC due to its particular characteristics, such as small size,
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278 121 relatively shallow average water depth, oligotrophy and high biological diversity,
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280 122 among others (Calvo et al., 2011).

283
284 123 The European sea bass, *Dicentrarchus labrax* (Linnaeus, 1758) (FAO, 2005) is one of
285
286 124 the most appreciated cultured fish species in the Mediterranean. Concerns are raised
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288 125 regarding its physiology and reproduction that could be compromised by the
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290 126 temperature increases predicted in a CC scenario (Almeida et al., 2015). Specifically,

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298 127 changes in water temperature are known to adversely affect a wide number of biological
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300 128 functions in this species including sex ratios, reproduction, growth, immune response,
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302 129 osmoregulatory capacity, xenobiotic biotransformation and antioxidant defences, among
303
304 130 others, making the fish more vulnerable to additional stressors (Almeida et al., 2015;
305
306 131 Samaras et al., 2018). Furthermore, metabolic alterations in muscle, liver and brain in
307
308 132 response to a 4 °C increase were enhanced after exposure to methylmercury in the
309
310 133 European sea bass, raising the possibility for a synergistic effect between both stressors
311
312 134 (Maulvault et al., 2017).

315 135 To the best of our knowledge, no studies have assessed potential toxic effects of fipronil
316
317 136 in *D. labrax*. However, this insecticide is known to act as endocrine disrupter in several
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319 137 other fish (Mnif et al., 2011; Bencic et al., 2013; Sun et al., 2014), to induce oxidative-
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321 138 stress due to reactive oxygen species (ROS) generation, and to interfere with a number
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323 139 of isoenzymes of the cytochrome P450 (CYPs) family, a main hepatic
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325 140 biotransformation route of this compound in different vertebrates (Wang et al., 2016).

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328 141 The aim of this study was to evaluate bioaccumulation, biotransformation and
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330 142 alterations in key physiological pathways of European sea bass after fipronil exposure
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332 143 in an environmentally-realistic scenario of temperature increase predicted for the NW
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334 144 Mediterranean region. The effects of fipronil dietary administration were assessed using
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336 145 a wide array of biomarkers encompassing several physiological and detoxification
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338 146 endpoints in different tissues and in two conservative matrices (i.e. plasma and skin
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340 147 mucus) in an effort to use them as non-lethal indicators of the effects of this pesticide in
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342 148 animal experimentation.

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347 150 **2. Material and Methods**

348 349 151 *2.1. Experimental design*

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356
357 152 Juvenile European sea bass (8 months old) were obtained from the Institute of Research
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359 153 and Technology Food and Agriculture (IRTA, Sant Carles de la Ràpita, Spain). Fish
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361 154 were transported and maintained at the Experimental Aquaria facilities (ZAE) of the
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363 155 Institute of Marine Sciences (ICM-CSIC, Barcelona, Spain). Prior to the experiment,
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365 156 fish were acclimated for a two-week period in a 2,500 L round fiberglass tank
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367 157 containing filtered sea-water (sterilized sand filter 50 μm) under natural conditions of
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369 158 temperature and with a water full-renovation rate of 24 times per day. Fish were fed
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371 159 daily *ad libitum* with commercial pellets (L-4 Optibass 2P, Skretting, Spain). After an
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373 160 initial two-week acclimation to lab conditions, fish were randomly assigned to four 600
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375 161 L round fiberglass tanks (19–20 individuals per tank). Two of them were reared at
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377 162 natural water temperature ($T \approx 13\text{ }^{\circ}\text{C}$) and the other two at $3\text{ }^{\circ}\text{C}$ above the natural
378
379 163 temperature ($T \approx 16\text{ }^{\circ}\text{C}$). The new experimental temperature was gradually attained at
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381 164 an increasing rate of $1\text{ }^{\circ}\text{C}$ per day and fish were acclimated to these new conditions for
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383 165 two additional weeks. Then, fish were fed a diet containing Regent®800WG (80%
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385 166 fipronil) at a concentration of active ingredient of 10 mg fipronil/Kg feed, which was
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387 167 prepared following the alcohol evaporation method adapted for sea bass (Blázquez et
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389 168 al., 1995; Blanco et al., 2016). Briefly, a monolayer of pelleted dry feed was carefully
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391 169 sprayed with the insecticide dissolved in 15 ml ethanol and the solvent was allowed to
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393 170 evaporate completely at room temperature and kept stored at $4\text{ }^{\circ}\text{C}$ until used. Fish were
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395 171 sampled just before the start of the experimental diet (t_0) and considered as control, and
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397 172 after 7 (t_7), and 14 (t_{14}) days of fipronil administration. At this point (t_{14}), fipronil
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399 173 treatment finished and fish were fed with non-spiked commercial feed (depuration
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401 174 period) for an extra week completing 21 days from the start of the experiment (t_{21}).
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403 175 Throughout the experiment, total feed consumption was quantified in each tank by
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405 176 initially weighing the amount before manual feeding and, when fish stopped feeding,
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416 177 weighing the remaining amount; consumption was then calculated by weight difference.
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418 178 During the experiment, values (mean \pm standard deviation) of physical water parameters
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420 179 were: Temperature = 13.37 ± 0.23 for the groups reared at natural temperature and
421
422 180 16.55 ± 0.44 °C for those reared at +3 °C. Other water parameters ranged as follows:
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424 181 dissolved O₂ = 6.66 ± 0.32 and 6.59 ± 0.47 mg/L (81% and 85% saturation,
425
426 182 respectively); pH = 7.73 ± 0.35 and 7.64 ± 0.31 ; salinity = 37.78 ± 0.12 and $37.93 \pm$
427
428 183 0.27 psu, for the 13 °C and 16 °C groups, respectively. During the experiment, fish were
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430 184 reared under natural photoperiod corresponding to 10 h light:14 h dark.
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435 186 *2.2. Fish Sampling*

437 187 Eight fish were sampled for each temperature regime (4 fish / replicate tank) just prior
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439 188 to the beginning of the exposure period (t0), after 7 (t7) and 14 (t14) days of fipronil
440
441 189 administration, and after 7 days of depuration (t21). Fish were fasted for 48 h before
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443 190 each sampling time. Fish were anesthetized with 0.2 % 2-phenoxyethanol (Sigma-
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445 191 Aldrich, St. Louis, MO, USA), measured (standard length: SL) and weighed (total body
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447 192 weight: BW). Epidermal mucus was collected on sterile glass slides from the over-
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449 193 lateral line in caudal direction with especial care to avoid contamination with blood
450
451 194 and/or urogenital and intestinal excretions (Fernandez-Alacid et al., 2018). Slides were
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453 195 gently wiped along both sides of the animal twice or three times, and mucus was
454
455 196 carefully kept in a 1.5 mL sterile tube, snap frozen in liquid nitrogen and stored at -80°C
456
457 197 until use. About 1 ml of blood was withdrawn from the caudal vein using heparinized
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459 198 syringes and kept on ice until centrifugation. Fish were sacrificed by severing their
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461 199 spinal cord, eviscerated, weighed (EW) and the weight of liver, gonads and visceral fat
462
463 200 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 $-80\text{ }^{\circ}\text{C}$ 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 $3,000\times g$ for
216 15 min at $4\text{ }^{\circ}\text{C}$). Mucus was homogenized using a sterile Teflon implement and
217 centrifuged at $14,000\times g$ for 15 min at $4\text{ }^{\circ}\text{C}$. Plasma and mucus supernatants were
218 aliquoted and stored at $-80\text{ }^{\circ}\text{C}$ for further metabolite and biochemical analyses.

219

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 $10,000\times g$ for
224 30 min at $4\text{ }^{\circ}\text{C}$ to obtain the S10 fraction. The supernatant was aliquoted and stored at $-$
225 $80\text{ }^{\circ}\text{C}$ for further biochemical determinations.

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536 227 *2.3.3. Liver*
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538 228 About 1.5 g of each liver were homogenized in ice-cold buffer phosphate (100 mM pH
539 229 7.4) containing 150 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM phenanthroline, 0.1
540 230 mg/mL trypsin inhibitor and 1 mM EDTA in a 1:4 (w:v) ratio using a Polytron®
541 231 blender. Homogenates were centrifuged at 10,000 ×g for 30 min at 4 °C to obtain the
542 232 S10 fraction, of which 1 mL was withdrawn while the rest was further homogenised at
543 233 100,000 ×g for 60 min at 4 °C to obtain microsomal and cytosolic fractions.
544 234 Microsomal pellets were dissolved in the above-described homogenization buffer, also
545 235 containing 20 % glycerol in a 2:1 (w:v) ratio (Crespo and Solé, 2016). S10, microsomal
546 236 and cytosol fractions were aliquoted and stored at –80 °C for further biochemical
547 237 determinations.
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551 239 *2.3.4. Kidney*
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553 240 About 0.05–0.1 g of each individual kidney were homogenised in ice-cold buffer (pH
554 241 7.3) containing 150 mM reagent-grade sucrose, 50 mM imidazole and 10 mM
555 242 Na₂EDTA in a 1:15 (w:v) ratio using a Polytron® blender. Homogenates were
556 243 centrifuged at 5,000 ×g for 2 min at 4 °C. The resulting supernatant was aliquoted and
557 244 stored at –80 °C for osmoregulation and enzymatic measures. A more detailed
558 245 description is given in González-Mira et al. (2018).
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560 246
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562 247 *2.3.5. Digestive tract*
563
564 248 The intestines were divided into anterior and posterior regions of equal length and in
565 249 each of them, pancreatic (trypsin, chymotrypsin, bile salt activated lipase and alpha-
566 250 amylase) and intestinal brush border (BB) enzymatic activities (alkaline phosphatase,

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592
593 251 aminopeptidase N and maltase) were quantified. Further methodological details can be
594
595 252 found elsewhere for pancreatic enzymes (Gisbert et al. 2009) and intestinal enzymes
596
597 253 (Gisbert et al. 2018). The activity of non-specific esterases was also determined in the
598
599 254 pancreatic fraction. Intestines from t7 group were discarded from the study because in
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601 255 this case fish were fasted only for 24 h, as opposed to 48 h in the other groups,
602
603 256 something that could affect the activity of digestive enzymes.
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608 258 *2.4. Biochemical analyses*

609
610 259 All reactions were carried out in triplicate at 25 °C, except for CYPs, UDPGT and
611
612 260 digestive enzymes determinations, which were measured at 30 °C on a Tecan™Infinite
613
614 261 M200 spectrophotometer.
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618 263 *2.4.1. Plasmatic and skin mucus metabolites and lysozyme determination*

619
620 264 Glucose and lactate content in plasma and skin mucus (expressed as µg/mL) were
621
622 265 determined by enzymatic colorimetric kit tests GOD-POD glucose (Ref: 41011) and
623
624 266 LO-POD lactate (Ref: 1001330), from SPINREACT® (Spain), according to the
625
626 267 methodology described in Fernández-Alacid et al. (2018).
627
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629 268 Plasmatic ammonia (expressed as µmol/L) was analyzed using a commercial kit by
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631 269 SPINREACT®, and (Ref: 1001410).
632

633 270 Lysozyme activity in plasma (expressed as units (U)/ mg of total plasmatic protein) was
634
635 271 measured according to the turbidimetric method described by Parry et al. (1965) with
636
637 272 some modifications. Briefly, 100 µL of plasma diluted in a 1:2 ratio with 10 mM PBS
638
639 273 pH 6.2 were placed in flat-bottomed 96-well plates. To each well, 100 µL of freeze-
640
641 274 dried *Micrococcus lysodeikticus* (0.3 mg/ml, Sigma) were added as lysozyme substrate.
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644 275 The absorbance ($\lambda = 450$ nm) was measured at the beginning and after 15 min. Units of
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652 276 lysozyme present in plasma were obtained from a standard curve built with chicken egg
653
654 277 white lysozyme (HEWL, Sigma).

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659 279 *2.4.2. Anaerobic metabolism*

660
661 280 Lactate dehydrogenase (LDH) activity was measured in the S10 fraction of the liver
662
663 281 following adaptation of the Vassault (1983) method using NADH (200 μ M) and
664
665 282 pyruvate (1 mM) as final well concentrations. Reading was done at $\lambda = 340$ nm for 5
666
667 283 min. LDH activity was expressed as nmol/min/mg total protein.

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672 285 *2.4.3. Oxidative-stress parameters*

673
674 286 Activities of the antioxidant enzymes catalase (CAT), total glutathione peroxidase
675
676 287 (GPX) and glutathione reductase (GR) were determined in the liver cytosolic fraction.
677
678 288 CAT activity was measured as a decrease in absorbance at $\lambda = 240$ nm using H_2O_2 (50
679
680 289 mM) as substrate; GPX and GR used cumene hydroperoxide (CHP, 0.625 mM) and
681
682 290 oxidized glutathione (GSSG, 0.9 mM) as respective substrates and NADPH as cofactor
683
684 291 in both assays at $\lambda = 340$ nm. Lipid peroxidation levels (LPO) were quantified in muscle
685
686 292 and in S10 liver fraction using a colorimetric method with 1-methyl-2-phenylindole.
687
688 293 Quantification, with respect to the standard solution 1,1,3,3-tetramethoxypropane, was
689
690 294 made at $\lambda = 586$ nm. CAT activity was expressed as μ mol/min/mg total protein and GR
691
692 295 and GPX activities as nmol/min/mg total protein and LPO levels as nmol MDA
693
694 296 (malondialdehyde)/g wet weight.

697
698 297

699
700 298 *2.4.4. Conjugation enzymes*

701
702 299 Glutathione *S*-transferase (GST) determination was performed in the liver cytosolic
703
704 300 fraction according to the method of Habig et al. (1974) using 1 mM GSH as substrate at

709
710
711 301 $\lambda = 340$ nm. Uridine diphosphate glucuronyltransferase (UDPGT) activity was
712
713 302 measured in liver microsomes according to the method of Collier et al. (2000) using
714
715 303 methyl umbelliferone (MU, 0.1 mM) as substrate in the fluorometric mode (EX/EM
716
717
718 304 355/460). Both activities were expressed as nmol/min/mg total protein.
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720 305 721 722 306 *2.4.5. CYP components and reductases*

723
724 307 Catalytic activities of hepatic CYPs were determined in the microsomal liver fraction
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726 308 using six fluorometric substrates: 7-ethoxyresorufin (ER), 7-benzyloxyresorufin (BR),
727
728 309 7-methoxyresorufin (MR), 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 3-cyano-7-
729
730 310 ethoxycoumarin (CEC) and 7-ethoxycoumarin (EC). Assay conditions were based on
731
732 311 the method by Solé et al. (2012). Briefly, microsomes (10 μ L) were incubated for 10
733
734 312 min at 30 °C and the metabolite formed was recorded at its specific wavelength (Smith
735
736 313 and Wilson, 2010). A calibration curve for each specific metabolite was done (range 0–
737
738 314 160 nM). CYPs assays were run in 100 mM phosphate buffer pH 7.4, except for ECOD
739
740 315 determination, which was done in 100 mM Tris buffer pH 7.4. Activities were
741
742 316 expressed in pmol/min/mg total protein.

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744
745 317 Microsomal reductases, NAD(P)H- cytochrome c reductases and NADH- ferricyanide
746
747 318 reductase activities, were measured by the increase in absorbance at $\lambda = 550$ nm and the
748
749 319 decrease in absorbance at $\lambda = 420$ nm, respectively (Solé and Livingstone, 2005). Assay
750
751 320 conditions were: 50 mM Tris-HCl buffer pH 7.6, 1 mM KCN, 0.26 mM NAD(P)H, and
752
753 321 60 μ M cytochrome c or 0.2 mM potassium ferricyanide. Sample volumes were: 10 μ L
754
755 322 microsomal fraction for NADPH- and 15 μ L for NADH-dependent reductases. Results
756
757 323 are expressed in nmol/min/mg total protein.
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760 324 761 762 325 *2.4.6. Osmoregulation*

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770 326 Activity of Na⁺/K⁺-ATPase was determined in the head kidney of fish following Zaugg
771
772 327 (1982) method with modifications (González-Mira et al., 2018). Sample absorbance was
773
774 328 measured at λ = 750 nm after 30 min incubation. Na⁺/K⁺-ATPase activities were
775
776 329 expressed as μmol ATP hydrolysed/mg total protein/hour. Plasmatic osmolality was
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778 330 measured with the aid of a Fiske® 210 Micro-Sample Osmometer using 20 μL plasma
780
781 331 and expressed in mosm/Kg H₂O.
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783 332

785 333 *2.4.7. Digestive enzymes*

786
787 334 The methods used for enzyme quantification are briefly described as follows: trypsin
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789 335 and chymotrypsin activities, the two main pancreatic alkaline proteases, were assayed
790
791 336 using, respectively, N-benzoyl-DL-arginine p-nitroanilide (BAPNA) (Holm et al., 1988)
792
793 337 and Succinyl-L-Ala-Ala-Pro-L-Phenylalanine p-nitroanilide (SAAPNA) (Erlanger et
794
795 338 al., 1961). Alpha-amylase activity was estimated using 2-chloro-p-nitrophenyl-α-D-
796
797 339 maltotrioside as substrate (Lorentz et al., 1999). The activities of bile-salt-activated
798
799 340 lipase and non-specific esterases were measured using p-nitrophenyl myristate (Iijima et
800
801 341 al., 1998) and p-nitrophenyl acetate (Hosokawa and Satoh, 2005) as respective
802
803 342 substrates. The activity of the alkaline phosphatase was determined using 4-
804
805 343 nitrophenylphosphate (Bessey et al., 1946), aminopeptidase N activity was determined
806
807 344 using L-leucine p-nitroanilide (Maroux et al., 1973) and maltase activity was
808
809 345 determined using d(+)-maltose (Dahkqvist, 1970) as substrates. All enzymatic activities
810
811 346 were expressed as specific units (mU/mg total protein).
812

813 347

817 348 *2.4.8. Protein determination*

827
828
829 349 Total protein content of all samples was determined by the Bradford method (1976)
830
831 350 using the Bio-Rad Protein Assay reagent and bovine serum albumin (BSA; 0.05-1
832
833 351 mg/mL) as standard. The absorbance was read at $\lambda = 595$ nm.
834
835
836 352

837 838 353 *2.5. Chemical analyses*

839 840 354 *2.5.1. Bile*

841
842 355 Bile glands from group t7 were mostly empty (as described in digestive enzyme
843
844 356 measures), and could not be used for chemical analysis. A more detailed methodology
845
846 357 (adapted from Aceña et al. (2017)), as well as reference standards and solvent solutions
847
848 358 characteristics and a description for fipronil and its metabolites quantification, is
849
850 359 reported as electronic supplementary material (ESM).

851
852 360 All analyses were performed using a SCIEX ExionLC™ AD system coupled to a hybrid
853
854 361 SCIEX X500R QTOF system (Sciex, Redwood City, CA, U.S.) equipped with a Turbo
855
856 362 V™ source and Electrospray Ionization (ESI).
857
858

859 363

860 861 364 *2.5.2. Muscle*

862
863 365 Analysis of fipronil in muscle was based on the use of the commercial brand
864
865 366 Regent®800WG (80% fipronil) as standard and following the solid phase QuEChERS
866
867 367 extraction method and gas chromatography-electron impact (GC-EI) detection at the
868
869 368 Scientific and Technological Centres of the University of Barcelona (CCiTUB) that
870
871 369 holds the quality standard ISO 9001:2015. A more detailed description of the analytical
872
873 370 procedure is provided as ESM.
874
875

876 371

877 878 372 *2.6. Data analyses*

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888 373 Fish hepatosomatic index (HSI) and gonadosomatic index (GSI) were calculated as
889
890 374 (liver weight/BW) \times 100 and (gonad weight/BW) \times 100, respectively. Fish condition
891
892 375 was assessed by Fulton's condition factor (CF), calculated as (BW/TL³) \times 100.
893
894 376 Daily feed intake was measured per tank and food consumption calculated in relation to
895
896
897 377 the total number of fish per tank. For each mucus sample, the ratios of glucose/lactate,
898
899 378 glucose/protein and lactate/protein were calculated.
900
901 379 A detailed explanation of statistical procedures is provided as ESM. In short,
902
903 380 relationships among fish biological and some biochemical variables and their
904
905 381 interactions were tested by Pearson' or Spearman' rank correlations (continuous
906
907 382 variables) and by Student's t-tests or Mann-Whitney U-tests (sex-related differences).
908
909 383 For digestive enzymes, differences between anterior and posterior parts of the digestive
910
911 384 tract were tested using Wilcoxon pairwise tests with repeated measurements.
912
913 385 Possible effects of treatment duration and temperature were tested by general or
914
915 386 generalized linear models (GLMs/GZMs) followed by Student's t-test/Mann-Whitney
916
917 387 U-test and one-way ANOVA/Kruskal-Wallis tests. A permutation multivariate analysis
918
919 388 (PERMANOVA) and a principal component analysis (PCA) were also carried out in
920
921 389 order to assess a global biochemical response to temperature and treatment duration In
922
923 390 all cases, significant differences were set at $p < 0.05$.
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925
926
927 391

928 392 **3. Results**

930 393 *3.1. Biometric parameters and general condition indices*

931 394 Mean values for fish biometric data and general condition indices were fairly uniform
932
933 395 across experimental groups (Table 1). Most individuals were immature, as evidenced by
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935 396 low GSI values in both sexes. Sex ratios were generally skewed, with more females
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947 397 than males in most groups. Significant increases of SL and BW with time were
948
949 398 observed ($\chi^2 = 10.589$, $p = 0.014$ and $\chi^2 = 8.613$, $p = 0.035$, respectively).

951 399 Direct correlations among biometric data and condition indices were found in most
952
953 400 cases, with visceral fat weight showing positive associations with all other biological
954
955 401 variables ($r_p = 0.299$ – 0.692 , $p = 0.018$ – < 0.001), in a similar way as BW (only non-
956
957 402 significant correlation to HSI) ($r_p = 0.268$ – 0.938 , $p = 0.032$ – < 0.001). SL and GSI were
958
959 403 also positively correlated ($r_p = 0.483$, $p < 0.001$), as well as CF and HSI ($r_p = 0.457$, $p <$
960
961 404 0.001). Body weight, GSI and visceral fat weight displayed higher values in females
962
963 405 than in males ($t = 2.690$ – 6.252 , $p = 0.012$ – < 0.001).

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965
966 406

967 407 *3.2. Feed consumption*

968
969 408 Fish feeding rate was similar at the two experimental temperatures: 0.54 ± 0.20 and 0.57
970
971 409 ± 0.22 g/fish at 13°C and 16°C , respectively, and significantly increased over time ($F_{(3,$
972
973 410 $24)} = 23.891$, $p < 0.001$) (Table 1).

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975
976 411

977 412 *3.3. Biochemical analyses*

978 413 *3.3.1. Plasmatic and skin mucus analyses*

979
980 414 Mean levels of plasmatic glucose ranged between 110.40 ± 14.20 and 212.30 ± 22.20
981
982 415 mg/dL and lactate between 52.70 ± 5.50 and 79.30 ± 7.10 mg/dL. In mucus these values
983
984 416 were much lower with glucose ranging between 0.57 ± 0.07 and 1.24 ± 0.11 mg/dL and
985
986 417 lactate between 0.42 ± 0.08 and 1.11 ± 0.14 mg/dL, (Table S1). Some statistical
987
988 418 correlations of the different parameters studied in plasma and skin mucus and most fish
989
990 419 biometrics and condition indices are detailed in Table S2.

991
992 420 Although no effect of the rearing temperature was found, a significant increase with
993
994 421 longer fipronil exposures was observed for glucose and lactate plasma levels ($F_{(3, 59)} =$

1004
1005
1006 422 7.098, $p < 0.001$ and $F_{(3, 53)} = 4.226$, $p = 0.009$, respectively) (Fig. 1A, B). Moreover, a
1007
1008 423 decrease of these biomarkers during the depuration period was observed in skin mucus
1009
1010 424 ($F_{(3, 59)} = 10.196$, $p < 0.001$ and $F_{(3, 59)} = 6.338$, $p = 0.001$, respectively) (Fig. 1C, D).
1011
1012 425 Glucose/protein and lactate/protein ratios (mean values $3.31 \pm 0.47 - 6.39 \pm 0.98 \mu\text{g}/\text{mg}$
1013 426 and $2.28 \pm 0.24 - 4.59 \pm 0.37 \mu\text{g}/\text{mg}$, respectively) in skin mucus were not affected by
1014
1015
1016
1017 427 temperature or fipronil exposure. However, glucose/lactate ratio ($1.06 \pm 0.12 - 1.95 \pm$
1018
1019 428 $0.29 \mu\text{g}/\text{mg}$) was higher at 13 °C than at 16 °C ($t = 2.733$, $p = 0.008$) (Table S1). A
1020
1021 429 significant interaction between exposure time and CF was found for lactate content in
1022
1023 430 mucus ($F_{(3, 54)} = 6.113$, $p = 0.001$). Strong positive correlations were detected among
1024
1025 431 protein, glucose and lactate both in plasma ($r_p = 0.411-0.632$, $p < 0.001$) and skin
1026
1027 432 mucus ($r_p = 0.434-0.706$, $p < 0.001$).

1029
1030 433 Plasmatic lysozyme activity ranged between 7.98 ± 2.13 and $13.24 \pm 2.53 \text{ U}/\text{mg}$ total
1031
1032 434 protein (Table S3) and was not affected by temperature or fipronil exposure time.
1033

1034 435

1036 436 3.3.2. Anaerobic metabolism

1037
1038 437 Mean LDH activity in liver ranged between 14.58 ± 1.42 and 16.25 ± 1.17
1039
1040 438 nmol/min/mg total protein (Table S3), with no association to fish biometric variables or
1041
1042 439 condition indices and it was not affected by water temperature or fipronil exposure.
1043

1044 440

1046 441 3.3.3. Oxidative-stress parameters

1047
1048
1049 442 Antioxidant enzymes GR ($5.56 \pm 1.05 - 7.98 \pm 0.84 \text{ nmol}/\text{min}/\text{mg}$ total protein), GPX
1050
1051 443 ($6.60 \pm 0.40 - 8.12 \pm 0.40 \text{ nmol}/\text{min}/\text{mg}$ total protein) and CAT ($63.84 \pm 10.27 - 111.12$
1052
1053 444 $\pm 68 \mu\text{mol}/\text{min}/\text{mg}$ total protein) (Table S3) were not affected by temperature, and only
1054
1055 445 CAT responded to fipronil exposure, decreasing after the depuration period (t21) ($F_{(3, 60)}$

1063
1064
1065 446 = 4.792, $p = 0.005$) (Fig. 2A). Regarding fish biological variables, only some negative
1066
1067 447 associations were observed between GR and some biological traits (Table S2).
1068
1069 448 Mean LPO levels ranged between 2.99 ± 0.74 and 6.21 ± 1.09 nmol MDA/g ww in
1070
1071 449 muscle and between 10.10 ± 1.74 and 17.35 ± 1.96 nmol MDA/g ww in the S10 liver
1072
1073 450 fraction (Table S3). In both cases, no effect of temperature was detected but a
1074
1075 451 significant increase in MDA equivalents during fipronil exposure was found in liver
1076
1077 452 ($F_{(3, 60)} = 7.436, p < 0.001$) (Fig. 2B).
1078
1079 453 Correlations among oxidative stress-related biomarkers are shown in Table 2, revealing
1080
1081 454 a similar trend for GR and GPX activities, while GR scaled negatively with CAT
1082
1083 455 activity and LPO levels in liver.
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1085
1086
1087 456

1088 457 *3.3.4. Conjugation enzymes*

1089
1090 458 Mean GST activity values ranged between 36.45 ± 2.43 and 51.05 ± 5.80 nmol/min/mg
1091
1092 459 total protein, and UDPGT between 0.75 ± 0.09 and 0.82 ± 0.08 nmol/min/mg total
1093
1094 460 protein (Table S3). Some positive correlations were detected between conjugation
1095
1096 461 enzymes and biological variables, and GST activity was higher in females (Table S2).
1097
1098 462 Both enzymes were unaffected by temperature and GST activity was significantly
1099
1100 463 enhanced with increasing fipronil exposure time ($F_{(3, 60)} = 4.245, p = 0.009$) (Fig. 2C).
1101
1102 464 GST activity displayed positive correlations with LPO in muscle and liver and was
1103
1104 465 negatively related to GR activity, while UDPGT activity was positively correlated with
1105
1106 466 CAT activity (Table 2).
1107
1108
1109
1110 467

1111 468 *3.3.5. CYP components and reductases*

1112
1113 469 EROD (mean activity = $15.33 \pm 1.31 - 25.35 \pm 3.86$ pmol/min/mg total protein), BROD
1114
1115 470 ($0.47 \pm 0.07 - 0.76 \pm 0.11$ pmol/min/mg total protein), MROD ($2.34 \pm 0.18 - 5.00 \pm$

1122
1123
1124 471 0.71 pmol/min/mg total protein), CECOD ($19.14 \pm 1.63 - 33.45 \pm 5.34$ pmol/min/mg
1125
1126 472 total protein) and ECOD ($3.35 \pm 0.66 - 9.42 \pm 1.47$ pmol/min/mg total protein)
1127
1128 473 activities (Table S3) displayed negative correlations with fish HSI, as well as negative
1129
1130 474 associations between MROD and visceral fat weight and between ECOD and CF (Table
1131
1132 475 S2). These same formerly mentioned activities were unaffected by temperature but
1133
1134 476 significantly decreased after fipronil exposure ($F_{(3, 60)} = 3.046, p = 0.036; F_{(3, 60)} =$
1135
1136 477 $4.327, p = 0.008; F_{(3, 60)} = 7.743, p < 0.001, F_{(3, 60)} = 3.795, p = 0.015$ and $F_{(3, 47)} =$
1137
1138 478 $3.211, p = 0.031$, respectively) (Fig. 2D, E). By contrast, BFCOD activity (62.91 ± 5.23
1139
1140 479 $- 98.97 \pm 22.34$ pmol/min/mg total protein) was not affected by temperature of fipronil
1141
1142 480 exposure (Table S3). Strong positive correlations were found among most CYPs and
1143
1144 481 between them and the oxidative-stress markers GR and GPX (Table 2). By contrast,
1145
1146 482 negative associations were observed between GST and MROD and between UDPGT
1147
1148 483 and most CYPs (Table 2).
1149
1150 484 In relation to CYP-dependent reductases (expressed in nmol/min/mg total protein):
1151
1152 485 NAD(P)H Cyt c reductase ($17.07 \pm 1.22 - 22.98 \pm 2.24$) and NADH-Cyt c ($22.79 \pm$
1153
1154 486 $3.36 - 31.97 \pm 5.34$) and ferricyanide reductases ($1006 \pm 95 - 1208 \pm 108$) (Table S3),
1155
1156 487 NADPH cyt c reductase displayed higher activity at the lowest temperature ($t = 2.575, p$
1157
1158 488 $= 0.012$) (Fig. 2F) and a positive weak correlation with fish visceral fat weight, and of
1159
1160 489 NADH Cyt c reductase, that was negatively associated to fish GSI (Table S2).
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1166

491 3.3.6. Osmoregulation

1169 492 Kidney Na^+/K^+ -ATPase mean activity ranged from 0.91 ± 0.11 to 1.94 ± 0.51 μ moles of
1170
1171 493 ATP hydrolysed/mg total protein/hour (Table S3), osmolality from 370.50 ± 4.69 to
1172
1173 494 400.50 ± 9.12 mosm/Kg H_2O and ammonia from 153.10 ± 9.46 to 333.62 ± 59.29
1174
1175 495 μ mol/L (Table S1). While Na^+/K^+ -ATPase activity and ammonia content did not
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1177
1178
1179
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1181
1182
1183 496 significantly vary as a function of temperature or experimental time, osmolality showed
1184
1185 497 a significant increase after fipronil exposure ($F_{(3, 57)} = 6.729, p = 0.001$).

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1188 498

1189 499 *3.3.7. Digestive enzymes*

1192 500 Significant differences in activity between anterior and posterior intestine sections were
1193
1194 501 detected for the pancreatic enzymes trypsin (t14 at 13 °C; $W = 36, p = 0.012$) and
1195
1196 502 chymotrypsin (t21 at 16 °C; $W = 27, p = 0.038$) and the BB enzymes alkaline
1197
1198 503 phosphatase (t21 at 13 and 16 °C; $W = 27, p = 0.039$ and $W = 28, p = 0.023$,
1199
1200 504 respectively), aminopeptidase N (t21 at 13 and 16 °C; $W = 33, p = 0.043$ and $W = 35, p$
1201
1202 505 $= 0.019$, respectively) and maltase (t0 at 16 °C and t21 at 13 and 16 °C; $W = 21-36, p =$
1203
1204 506 $0.012-0.046$). In light of these results, data from digestive enzymes for anterior and
1205
1206 507 posterior intestine regions were treated separately.

1208
1209 508 Regarding pancreatic enzymes (in mU/mg total protein) in anterior intestine: trypsin
1210
1211 509 ($45.60 \pm 3.07 - 61.20 \pm 2.63$), chymotrypsin ($61.60 \pm 15.87 - 104.70 \pm 33.26$), bile salt-
1212
1213 510 activated lipase ($21.50 \pm 3.73 - 42.70 \pm 11.33$), alpha-amylase ($4.40 \pm 0.92 - 10.50 \pm$
1214
1215 511 3.01) and non-specific esterases ($415.60 \pm 38.22 - 564.40 \pm 35.19$) (Table S4), lower
1216
1217 512 activities were observed for trypsin before fipronil exposure (t0) and after depuration
1218
1219 513 period (t21) ($F_{(2, 41)} = 5.193, p = 0.01$) (Fig. 2G), and for lipase and alpha-amylase over
1220
1221 514 time ($F_{(2, 44)} = 3.435, p = 0.042$ and $F_{(2, 45)} = 3.708, p = 0.033$, respectively). For
1222
1223 515 posterior intestine: trypsin ($45.70 \pm 3.22 - 64.40 \pm 10.03$), chymotrypsin ($117.30 \pm$
1224
1225 516 $39.39 - 428.50 \pm 158.87$), lipase ($24.90 \pm 3.82 - 30.80 \pm 3.94$), alpha-amylase ($3.10 \pm$
1226
1227 517 $0.44 - 9.70 \pm 2.48$) and non-specific esterases ($447.40 \pm 43.69 - 587.70 \pm 56.37$) no
1228
1229 518 significant trends were detected. In relation to intestinal BB enzymes (in mU/mg total
1230
1231 519 protein) in anterior intestine: alkaline phosphatase ($742.40 \pm 116.31 - 1,177.40 \pm$
1232
1233 520 284.87), aminopeptidase N ($117.30 \pm 22.32 - 220.50 \pm 22.80$) and maltase ($42.90 \pm$

1240
1241
1242 521 8.63 – 250.10 ± 50.02), higher activities were found for aminopeptidase N at 13 °C ($t =$
1243
1244 522 2.310, $p = 0.026$) and for maltase with time exposure ($F_{(2, 45)} = 34.975, p < 0.001$) (Fig.
1245
1246 523 2H). Among the enzymes assessed in posterior intestine: alkaline phosphatase ($540.80 \pm$
1247
1248 524 $109.19 - 826.20 \pm 175.18$), aminopeptidase N ($93.50 \pm 11.08 - 178.90 \pm 27.34$) and
1249
1250
1251 525 maltase ($26.20 \pm 2.92 - 158.20 \pm 23.95$), only maltase activity increased over time ($F_{(2,$
1252
1253 526 $45)} = 28.828, p < 0.001$).

1254
1255 527 Regarding to association of digestive enzymes with fish biometric variables and general
1256
1257 528 condition indices, few significant associations were found (Table S2) while mostly
1258
1259 529 positive correlations were found among intestinal enzymes (Tables S5 and S6).
1260

1261 530

1262 531 *3.4. Chemical analyses on bile and muscle*

1263
1264 532 Concentrations of fipronil and fipronil-sulfone in bile and in muscle (only fipronil) of
1265
1266 533 the different experimental groups are shown in Table 3. In addition, a more detailed
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1268 534 report on chemical results is provided as ESM.
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1270 535

1271 536 *3.5. Multivariate analyses*

1272
1273
1274 537 PERMANOVA analyses showed no effect of temperature but a significant influence of
1275
1276 538 fipronil on fish general biochemical profile ($Pseudo-F_{(3, 60)} = 2.790, p_{(perm)} = 0.0001$;
1277
1278 539 9876 unique permutations, all pairwise comparisons significant except those comparing
1279
1280 540 t7 and t14, and t14 and t21).

1281
1282
1283 541 Two-dimensional PCA plots represented 56.1 % of total variance on the first two
1284
1285 542 components, and 51.9 % of the total variance on the first and third components (Figure
1286
1287 543 3). These results suggest a differentiation according to the exposure time to fipronil
1288
1289 544 along the first axis, with samples corresponding to unexposed fish (t0) (Fig. 3A, right
1290
1291 545 part of the plot) clearly separated from the rest (Fig. 3A, left part of the plot), and
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1300
1301 546 according to temperature along the third axis (Fig. 3B). Pearson correlations indicated
1302
1303 547 associations between some biochemical markers and fish groups, namely between most
1304
1305 548 CYP-related activities and unexposed fish (t0), between LPO levels, plasmatic
1306
1307 549 metabolites and osmolality and fish from t7 and t14, and between LDH, NADPH-Cyt c
1308
1309 550 reductase and GST and fish from t14 and t21.
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1312 551

1313 1314 552 **4. Discussion**

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1316 553 The present study reports, for the first time, the effects of dietary fipronil exposure on
1317
1318 554 several physiological parameters of the European sea bass. The use of a comprehensive
1319
1320 555 set of biomarkers encompassing different physiological and detoxification processes
1321
1322 556 allows for the assessment of the effects of this pesticide on the health and general
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1324 557 condition of an economically important cultured fish species. Moreover, the combined
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1326 558 effects of fipronil and a 3 °C temperature increase (as predicted for the NW
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1328 559 Mediterranean region by the end of this century) constitute a novel approach to assess
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1330 560 the consequences of CC for the harmful effects of this chemical in this commercial fish
1331
1332 561 species widely used for human consumption.
1333
1334 562 Morphometric markers and condition indices remained unchanged regardless of fipronil
1335
1336 563 exposure or of the rearing temperature, suggesting that the 3 °C temperature variation,
1337
1338 564 fipronil concentration and/or the time of exposure assessed were below threshold limits
1339
1340 565 to affect them. The observed increase in feeding rate over time regardless of the
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1342 566 temperature likely accounts for higher feed consumption as fish increased in size, and
1343
1344 567 not to fipronil exposure, since no changes occurred during depuration.
1345
1346 568 Despite of the wide array of biochemical markers assessed in this study, the use of
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1348 569 multivariate tools helped to infer some general patterns in their response to the different
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1350 570 experimental conditions. It appears that temperature induced changes in some metabolic
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1360 571 parameters as shown by the segregation along the third PCA axis of fish reared at 13 °C
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1362 572 and 16 °C but the PERMANOVA analyses indicated that the temperature-induced
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1364 573 metabolic changes observed were not that clear. An integrated multi-biomarker
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1366 574 response to fipronil exposure, according to both multivariate analyses, showed that the
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1368 575 most prominent changes took place between unexposed (t0) and exposed (t7, t14 and
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1371 576 t21) fish. Notably, biochemical patterns after depuration (t21) clustered with those for
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1373 577 t14 fish both in PERMANOVA analysis and PCA plots, rather than with non-exposed
1374
1375 578 groups (t0) suggesting that the 7-days depuration period was too short to allow for the
1376
1377 579 full recovery of pre-exposure levels.
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1379 580 The choice of a modest temperature increase, 3 °C with respect to the group reared at
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1381 581 natural temperature, was considered as environmentally realistic under the IPCC
1382
1383 582 forecasts by year 2100, although sharper increases may occur in estuarine and coastal
1384
1385 583 ecosystems (IPCC, 2014) inhabited by European sea bass, particularly in their juvenile
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1387 584 stage. The generalized lack of effects of this temperature increase on the assessed
1388
1389 585 biomarkers contrasts with other studies performed on juveniles of this species, which
1390
1391 586 reported behavioural, physiological and biochemical changes although under warmer
1392
1393 587 conditions that could account for the different results (Vinagre et al., 2012; Almeida et
1394
1395 588 al., 2015). Furthermore, a synergic effect between warmer conditions and fipronil
1396
1397 589 exposure did not occur in the present study. However, one must keep into account that a
1398
1399 590 more realistic simulation of CC conditions, including alterations of other abiotic
1400
1401 591 variables (e.g. salinity, pH), could yield a different outcome.
1402
1403 592 In the present study, several parameters in plasma and mucus were included as potential
1404
1405 593 non-lethal indicators of fish stress condition. Glucose and lactate increases in plasma
1406
1407 594 could be a result of the mobilisation of energetic resources induced by higher metabolic
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1409 595 demands, especially during the depuration period. In turn, the concomitant drop in
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1418
1419 596 mucus could be explained by the need to spare energy when energetic demands
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1421 597 increase, as described in gilthead sea bream during a 2-weeks starvation period
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1423 598 (Fernández-Alacid et al., 2018) or under chronic cold temperature conditions (Sanahuja
1424
1425 599 et al., 2019). Thus, plasma and mucus metabolite levels do not necessarily match under
1426
1427 600 chronic or sustained stress conditions (several days-weeks), as it is observed in the
1428
1429 601 present study and contrary to what has been reported under acute stress (hours)
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1431 602 (Fernández-Alacid et al., 2019).
1432
1433 603 In fish, fipronil metabolism takes place by oxidation and reduction reactions catalysed
1434
1435 604 by cytochrome P450-related enzymes (CYPs), which generate different fipronil
1436
1437 605 metabolites (mainly fipronil sulfone) (Konwick et al., 2006; Wang et al., 2016; Li et al.,
1438
1439 606 2018). These metabolites can be even more toxic to insects, mammals, aquatic
1440
1441 607 organisms and birds than the parental compound (Leghait et al., 2009; Tavares et al.,
1442
1443 608 2015). In the present study, this important phase I metabolic pathway was assessed by
1444
1445 609 using several fluorometric substrates indicative of several CYP isoforms (Smith and
1446
1447 610 Wilson, 2010; Solé et al., 2014) and the general electron donors NAD(P)H Cyt c and
1448
1449 611 NADH ferricyanide reductases. The responses of CYP1A- and CYP2B-related
1450
1451 612 activities (EROD, BROD, MROD, ECOD and CECOD) showed a similar trend,
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1453 613 markedly decreasing after fipronil administration, suggesting certain overlapping
1454
1455 614 substrate specificity, whereas CYP3A4-associated BFCOD activity displayed the
1456
1457 615 opposite trend. The effects of fipronil on CYP-related activities is controversial; for
1458
1459 616 instance, *in vitro* studies with human hepatocytes (Das et al., 2006) suggested an
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1461 617 increase in CYP1A1-related activity at low concentrations (1 μ M), and a decrease at
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1463 618 higher ones (10 and 25 μ M). In the present study, ECOD activity was clearly inhibited
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1465 619 after 14 days of fipronil exposure and remained low even at the end of the depuration
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1467 620 period. This CYP-related activity was the only one responding to waterborne fipronil
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1478 621 exposure in the zebrafish, *Danio rerio*, showing a dose-dependent induction in several
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1480 622 tissues 24h after exposure (Wu et al., 2014). A recent study in the Caspian kutum fish,
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1482 623 *Rutilus kutum*, showed a strong correlation between *cyp1a* gene expression and different
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1485 624 antioxidant responses in several tissues, including liver (Ardeshir et al., 2018). The
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1487 625 study suggested that the increase of *cyp1a* gene expression after intraperitoneal (IP)
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1489 626 fipronil injection could be due to structural similarities between this compound and
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1491 627 some aryl hydrocarbon receptor agonists, such as halogenated hydrocarbons (Ardeshir
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1493 628 et al., 2018). In fact, fipronil transformation into more toxic metabolites fipronil-sulfide
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1495 629 and fipronil-sulfone is linked to oxidative stress (Wang et al., 2016). This is supported
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1497 630 by the present results, since CAT, the antioxidant enzyme, was inhibited after the
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1499 631 longest exposure to fipronil, supporting the notion that the production of oxyradicals
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1501 632 may overwhelm the protective capacity of this enzyme (Regoli and Giuliani, 2014).
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1503 633 Moreover, the increase of LPO levels in liver and muscle clearly confirmed a scenario
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1505 634 of oxidative damage to cell membrane lipids, most likely due to ROS generation as
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1507 635 previously suggested (Wang et al., 2016). In addition to the role of GST catalysing the
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1509 636 conjugation of glutathione with xenobiotics for detoxification purposes, other GST
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1511 637 isoforms appear implicated in the reduction of lipid hydroperoxides produced by ROS
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1513 638 (Regoli and Giuliani, 2014). Present data point at this possibility, as suggested by the
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1515 639 progressive increase in GST activity after fipronil exposure. Nonetheless, this increase
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1517 640 in antioxidant protection, as indicated by the positive correlations between GST activity
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1519 641 and LPO levels in liver and muscle was not enough to prevent the occurrence of
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1521 642 oxidised lipids even after depuration. Concordantly, several studies performed in fish
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1523 643 also reported the occurrence of oxidative-stress after fipronil administration, either
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1525 644 waterborne exposure (Clasen et al., 2012; Menezes et al., 2016; Ghazanfar et al., 2018),
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1527 645 after IP injection (Ardeshir et al., 2017a) and even considering a combination of
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1537 646 waterborne exposure and IP injection (Ardeshir et al., 2017b). Thus, induction of
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1539 647 oxidative stress is a well-accepted consequence of fipronil exposure.
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1541 648 Since the European sea bass is a euryhaline fish that inhabits waters with broad salinity
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1543 649 gradients during its life cycle, osmoregulation constitutes a key physiological process
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1545 650 worth to be considered. In the present study, two osmoregulation-related parameters
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1547 651 were assessed. A significant increase in plasmatic osmolality evidenced a reduced
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1549 652 capability of hypo-osmoregulation in seawater after fipronil administration. However,
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1551 653 another parameter also indicative of osmoregulation capacity such as kidney Na^+/K^+
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1553 654 ATPase activity was not affected. At this stage, we can only speculate that another
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1555 655 response on this parameter might have been obtained if it had been measured in gills
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1557 656 instead of kidney, since this marker seems to display a tissue-dependent pattern of
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1559 657 activity (Vargas-Chacoff et al., 2009). Consequences for an osmoregulatory imbalance
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1561 658 after fipronil exposure are particularly significant in this species, given that during early
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1563 659 life stages it inhabits estuarine and freshwater ecosystems potentially subjected to waste
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1565 660 water discharges from nearby agricultural areas where the insecticide may be used.
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1567 661 In the present study, the consequences of fipronil exposure were also evaluated in the
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1569 662 digestive system because of the high importance of growth and energy assimilation in
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1571 663 this cultured fish species. The effects of temperature and fipronil on the activity of the
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1573 664 main digestive pancreatic and BB enzymes were evaluated in proximal and distal
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1575 665 intestinal parts since fish intestine is characterized by proximo-distal gradients of
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1577 666 hydrolases (Xiong et al., 2011; Izvekova et al., 2013). In the present study, the decrease
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1579 667 on lipase activity in bile might be due to the presence of fipronil-derived compounds in
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1581 668 bile, such as fipronil sulfone, that could have impaired lipase activity. Similarly, the
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1583 669 pyretroid insecticide deltamethrin, used in combination with fipronil (Jiang et al., 2014),
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1585 670 has been shown to inhibit lipase activity in several fish species (Simon et al., 1999;

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1596 671 Gunes and Yerli, 2011). Regarding the glucosidases alpha-amylase and maltase,
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1598 672 suppression of the activity of the former has been reported in several fish species
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1600 673 exposed to different pollutants, and mostly explained by a reduction in substrate affinity
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1602 674 (Filippov et al., 2013). The opposite trend was observed for maltase activity in anterior
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1604 675 and posterior intestine regions after fipronil exposure. This is in line with the random
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1606 676 effects of toxics on glucosidases ranging from inhibition to stimulation depending on
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1608 677 toxicant concentration, its interaction with other chemicals and exposure time (Filippov
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1610 678 and Golovanova, 2012; Filippov et al., 2013). Therefore, the response of glucosidases to
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1612 679 fipronil (whose effects on digestive enzymes have never been addressed before) needs
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1614 680 to be further investigated before more consistent conclusions could be drawn. Similarly,
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1616 681 the effect of organic pollutants on proteolytic activity (i.e. trypsin) seems inconsistent;
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1618 682 while it significantly increased in the present study after fipronil administration, it
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1620 683 decreased in roach (*Rutilus rutilus*) exposed to polychlorinated biphenyls (PCBs)
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1622 684 (Golovanova et al., 2011) and it was unaffected by naphthalene (a polycyclic aromatic
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1624 685 hydrocarbon) in Mozambique tilapia, *Oreochromis mossambicus*, (Kuz'mina et al.,
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1626 686 1999). Since exposure to fipronil did not result in a decrease of BB enzymes, as
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1628 687 indicative of harm to enterocytes integrity (Lalles, 2010), it seems that no damage
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1630 688 occurred to intestinal epithelium at the tested concentration.
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1633 689 Chemical analyses in muscle and bile confirmed intake and clearance of fipronil during
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1635 690 the exposure period as well as a bioaccumulation trend over time and a depuration after
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1637 691 withdraw from diet. Higher levels of the metabolite fipronil-sulfone than those of the
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1639 692 parental fipronil in fish bile at t14 and t21 confirmed a metabolisation of the insecticide
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1641 693 within a few days. Notably, in rainbow trout, *Oncorhynchus mykiss* (Konwick et al.,
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1643 694 2006) and Nile tilapia, *Oreochromis niloticus*, (Li et al., 2018), fipronil-sulfone was
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1645 695 detected as soon as one day after exposure to the parent compound, indicating its rapid
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1655 696 biotransformation. In the present study, the parent compound fipronil was not detected
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1657 697 in the muscle and very low concentrations were quantified in bile at the end of the 7-day
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1659 698 depuration period (t₂₁). This is in agreement with the rapid elimination reported by
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1661 699 Konwick et al. (2006), who could not detect the pesticide in trout muscle 4 days after
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1663 700 the end of the exposure. In contrast, fipronil-sulfone persisted in bile after the
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1665 701 depuration period (7 days after the end of fipronil administration) at fairly high
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1667 702 concentrations, which may be related to its affinity towards organic carbon supporting
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1669 703 the view that fatty organs, such as liver, can act as a reservoir for fipronil residues (Li et
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1671 704 al., 2018; Qu et al., 2018). Indeed, fipronil-sulfone is considered to be more toxic to
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1673 705 aquatic species than fipronil itself (EPA, 1996). This was also confirmed in the present
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1675 706 study by modulation of the activities: reduced CAT, enhanced GST and LPO
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1677 707 occurrence even at the end of the depuration period. Some studies in fish have alerted
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1679 708 for the high bioaccumulation potential of fipronil-sulfone compared to the parent
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1681 709 compound (Konwick et al., 2006; Wang et al., 2016). Moreover, our results may also be
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1683 710 suggestive of a longer persistence of fipronil-sulfone with increasing temperatures, as
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1685 711 higher concentrations of this metabolite were present in the bile of European sea bass
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1687 712 reared at 16 °C than in those reared at 13 °C However, more studies are needed to
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1689 713 strengthen this hypothesis. If confirmed, a potential synergistic interaction between
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1691 714 fipronil-sulfone and warmer temperatures should be taken into consideration when
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1693 715 predicting future consequences in a global warming scenario.
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1700 717 **Conclusions**

1701
1702 718 A two-week dietary administration of the pesticide fipronil induced physiological
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1704 719 responses in the European sea bass, as indicated by alterations in several markers.
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1706 720 Trends on plasma and skin mucus metabolites were indicative of an increased energy
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1714 721 demand during fipronil exposure and after depuration. Fipronil administration also
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1716 722 caused an oxidative-stress condition that persisted even after depuration and was
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1718 723 accompanied by the modification of some phase I CYP-related activities and an increase
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1720 724 of phase II GST activity. Osmoregulation and some digestive enzymes were also altered
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1722 725 as a consequence of the pesticide administration. Chemical analyses in bile and muscle
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1724 726 confirmed intake and clearance of fipronil (faster in muscle than in liver) but persistence
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1726 727 of the metabolite fipronil-sulfone in bile even after the depuration period. Although a
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1728 728 modest temperature increase of 3 °C did not enhance fipronil effects, the persistence of
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1730 729 fipronil-sulfone in bile at higher temperature may alert for potential synergistic effects
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1732 730 in a CC scenario.
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1758 742 **Conflict of interest**

1759 743 The authors of the present study declare that they have no conflict of interest.
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Tables

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

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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*

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.

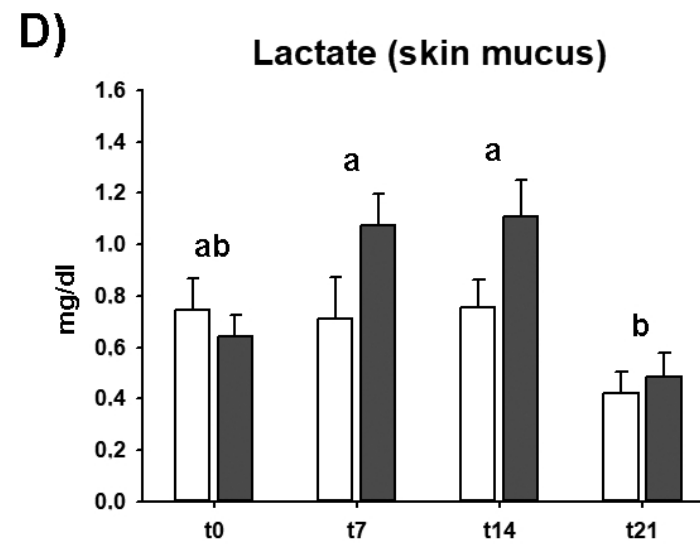
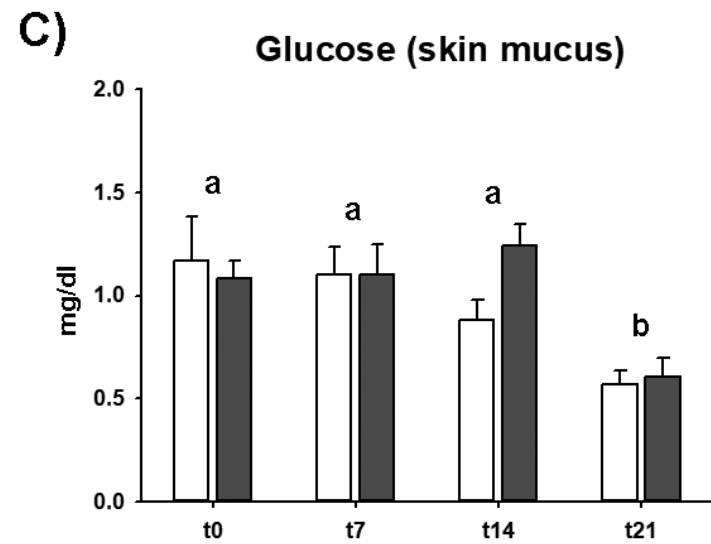
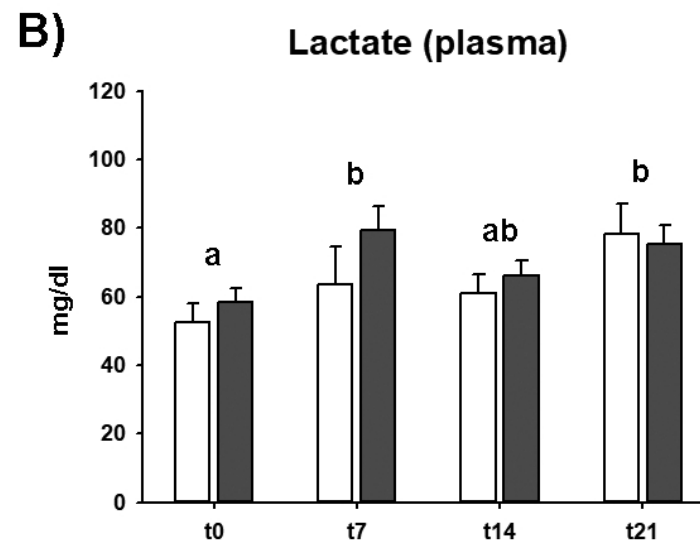
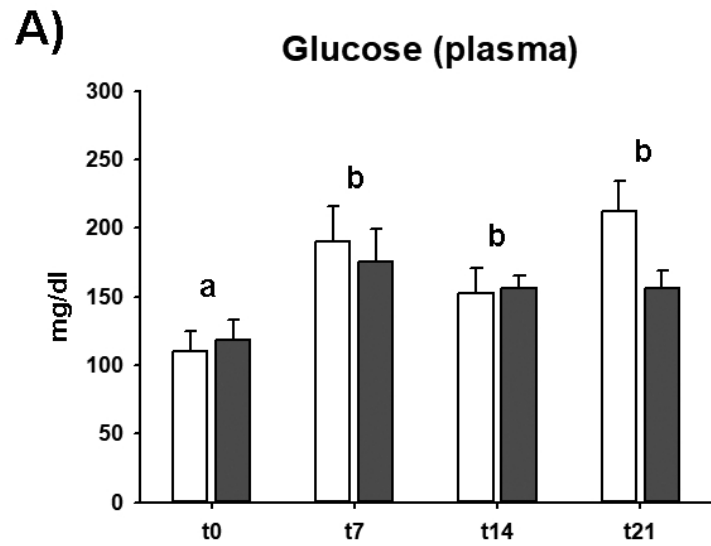
T °C	Time	Muscle	Bile	
		Fipronil (ng/g)	Fipronil (ng/ml)	Fipronil sulfone (ng/ml)
13 °C	t0	< LOD	< LOD	< LOD
	t7	88.4	–	–
	t14	64.6	4.08 ± 7.35	10.84 ± 9.02
	t21	< LOD	0.76 ± 0.47	15.17 ± 8.03
16 °C	t0	< LOD	< LOD	< LOD
	t7	42.9	–	–
	t14	57.7	4.70 ± 4.65	38.43 ± 20.16
	t21	< LOD	0.63 ± 0.62	34.58 ±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

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2681 **Figure captions**
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2683 **Figure 1.** Histograms displaying glucose and lactate levels in skin mucus (A and B,
2684 respectively) and plasma (C and D, respectively) of European sea bass exposed to
2685 fipronil under two temperature regimes before exposure (t0) after 7 and 14 days of
2686 exposure (t7 and t14, respectively) and after a 7-day depuration period following
2687 exposure (t21). Different letters show differences across temporal replicates (One-way
2688 ANOVA, $p < 0.05$). No differences between temperatures were detected.
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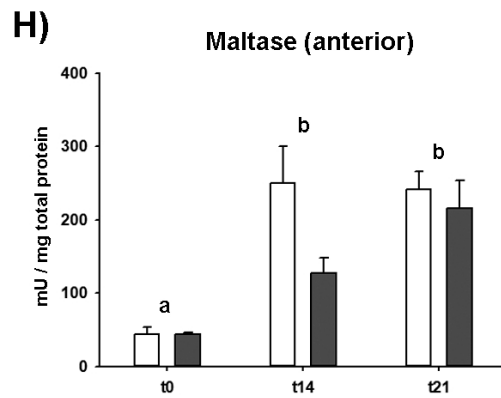
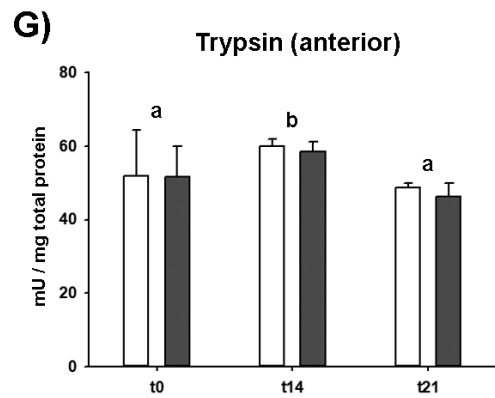
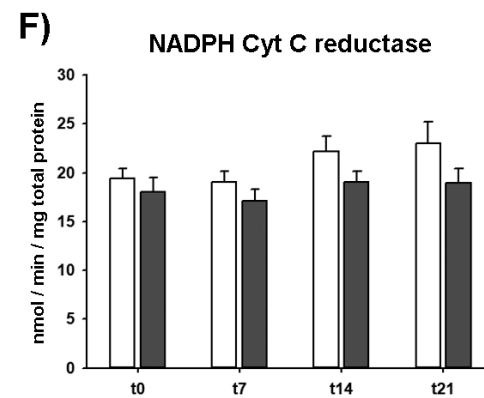
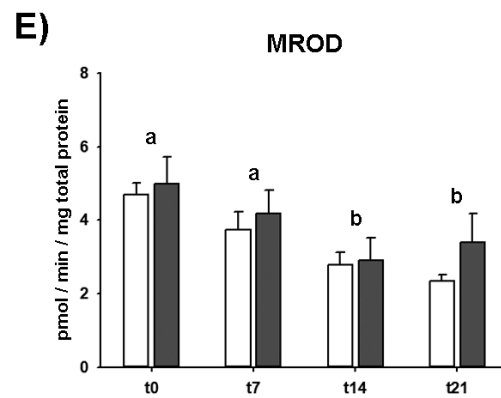
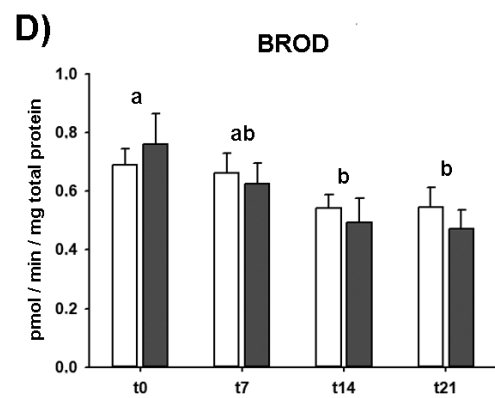
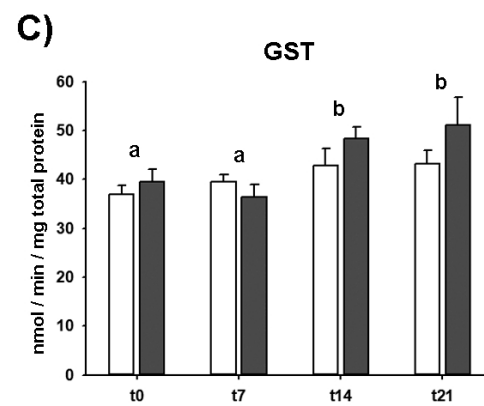
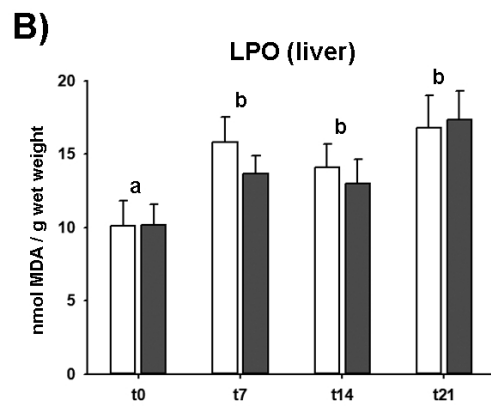
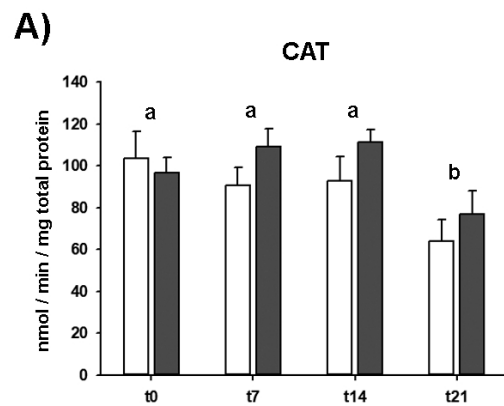
2690 **Figure 2.** Histograms displaying lipid peroxidation levels (LPO) (B) and activity levels
2691 of the enzymes catalase (CAT, A), glutathione-*S*-transferase (GST, C), Cytochrome
2692 P450-related BROD and MROD (D and E, respectively), NADPH Cytochrome C
2693 reductase (F), trypsin (G) and maltase (H) (the two latter in anterior intestinal region) in
2694 different tissues of European sea bass exposed to fipronil under two temperature
2695 regimes before exposure (t0) after 7 and 14 days of exposure (t7 and t14, respectively)
2696 and after a 7-day depuration period following exposure (t21). Different letters show
2697 differences across temporal replicates (One-way ANOVA, $p < 0.05$). No differences
2698 between temperatures were detected, except for NADPH Cyt C reductase.
2699

2700 **Figure 3.** Plots showing first and second components (A) and first and third
2701 components (B) of the principal components analysis (PCA) applied on biochemical
2702 data of European sea bass exposed to fipronil under two temperature regimes (filled
2703 symbols, 13 °C; empty symbols, 16 °C) before exposure (triangles, t0) after 7 and 14
2704 days of exposure (squares, t7 and circles, t14) and after a 7-day depuration period
2705 following exposure (rhombus, t21). Fish data were grouped according to combination of
2706 temperature and time conditions. Vectors represent Pearson' correlations between each
2707 variable and the PCA axis. The outer circle represents a correlation = 1.
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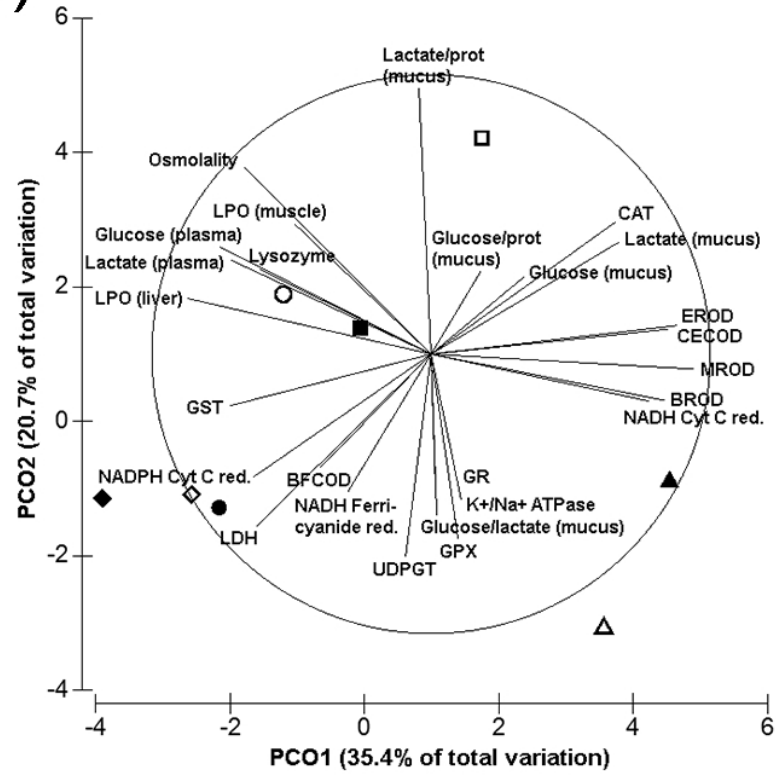
□ 13 °C

■ 16 °C

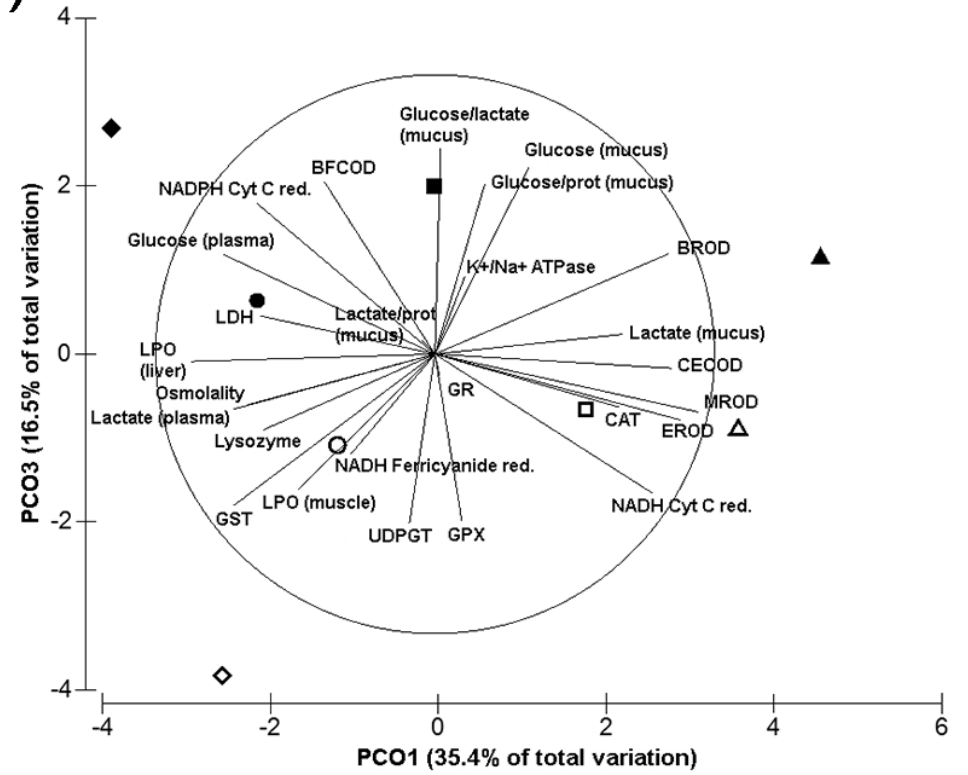


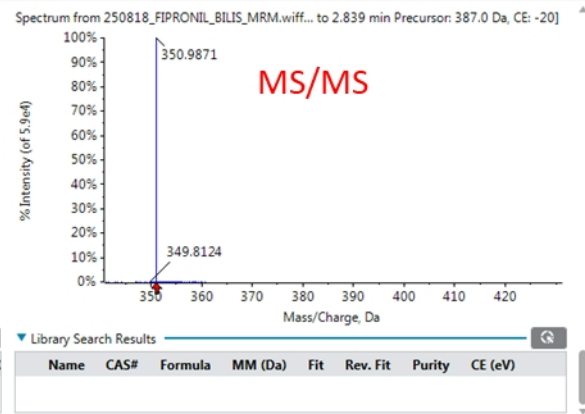
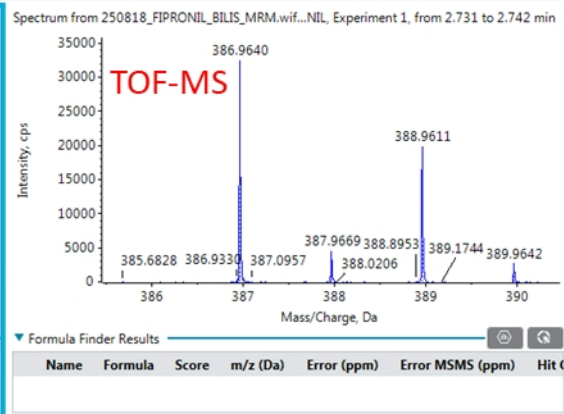
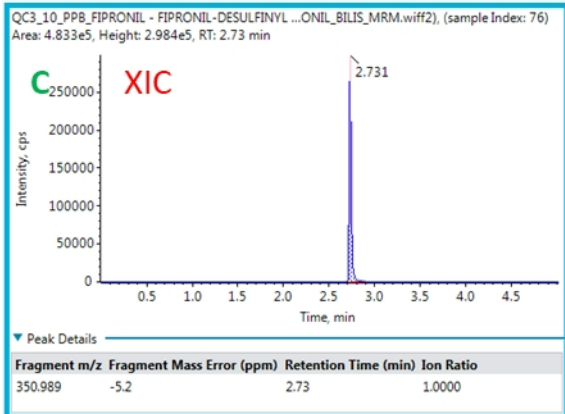
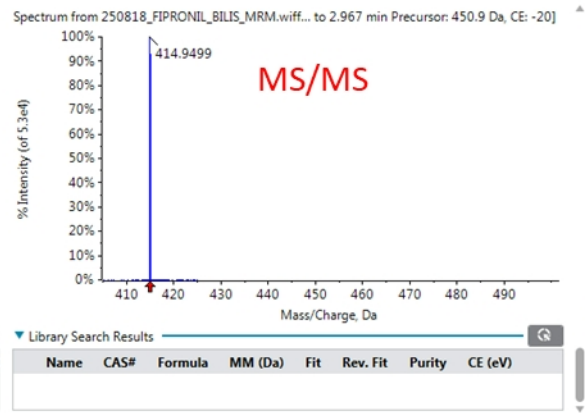
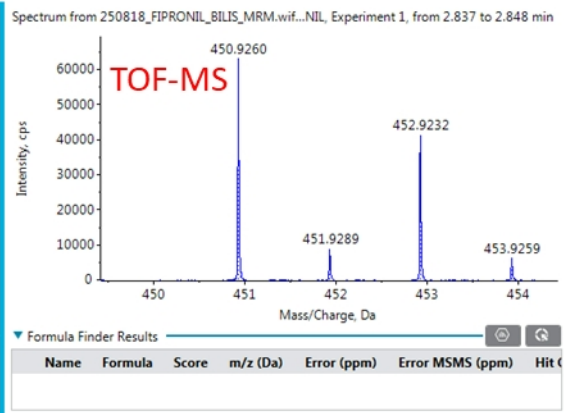
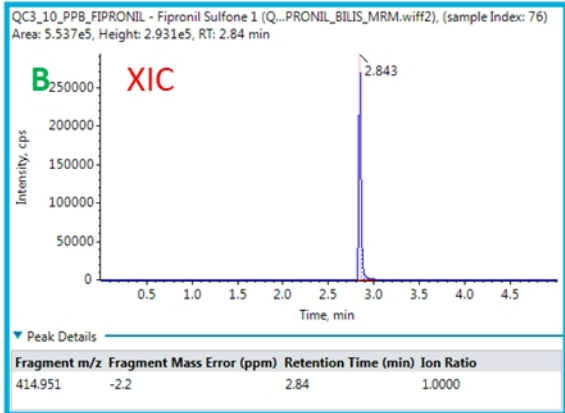
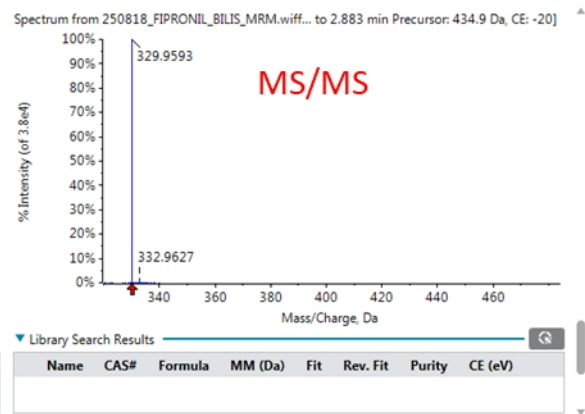
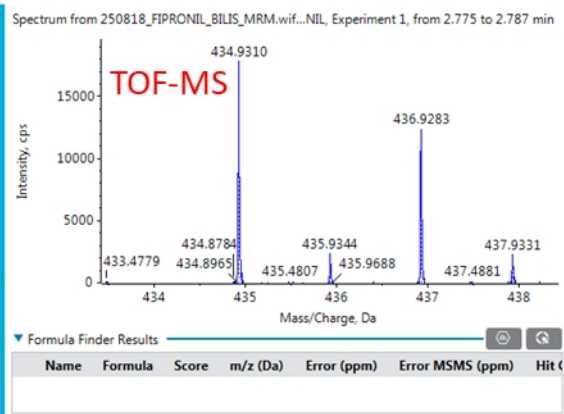
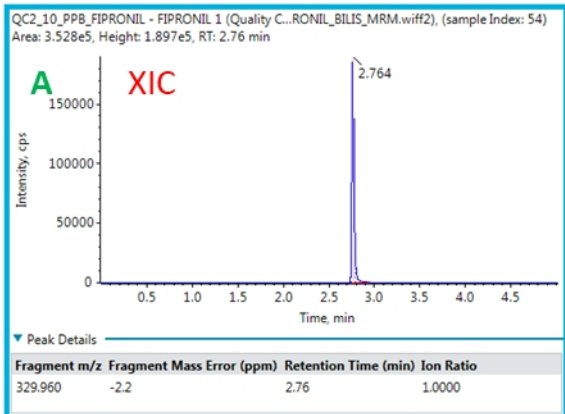
□ 13 °C
 ■ 16 °C

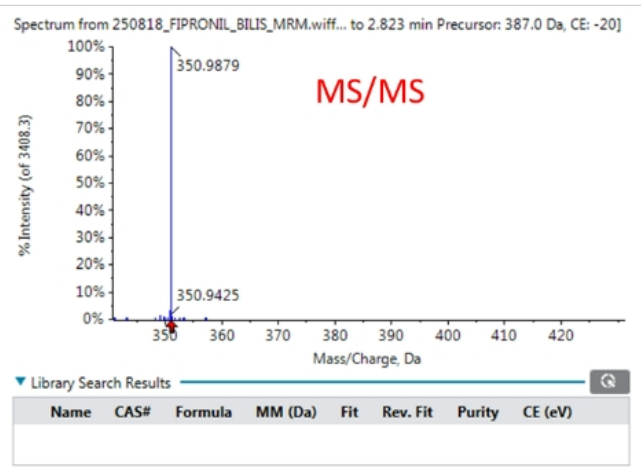
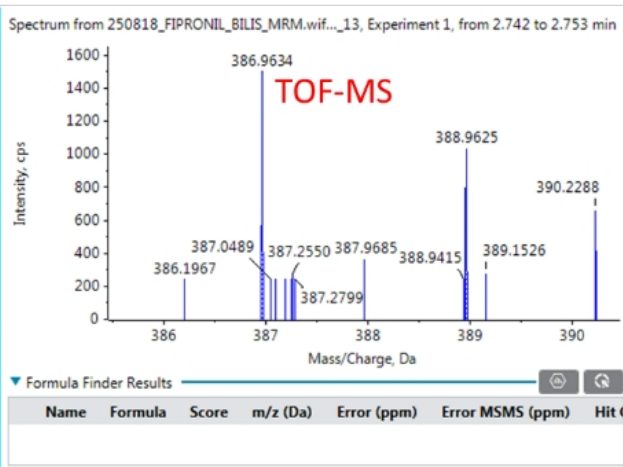
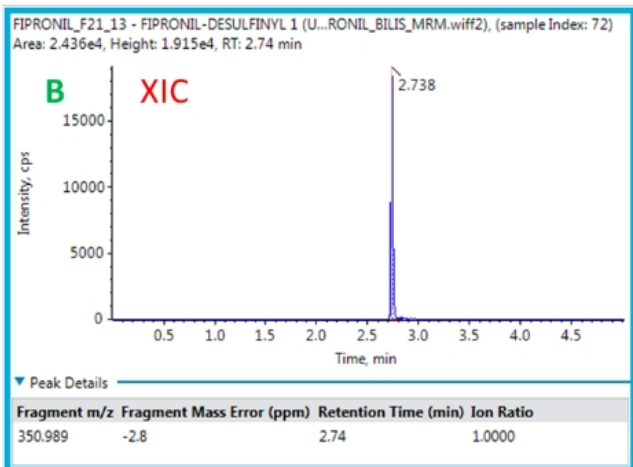
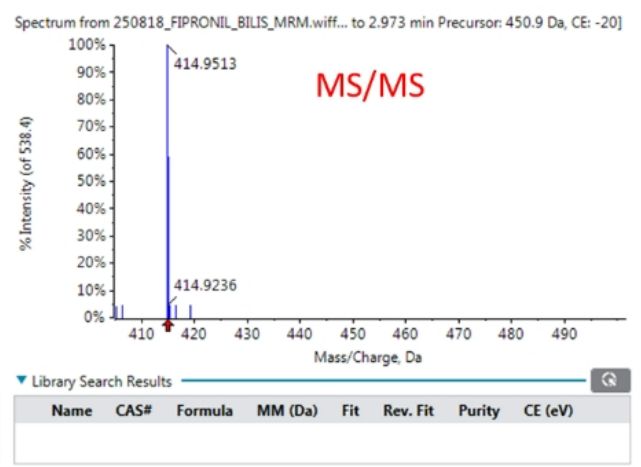
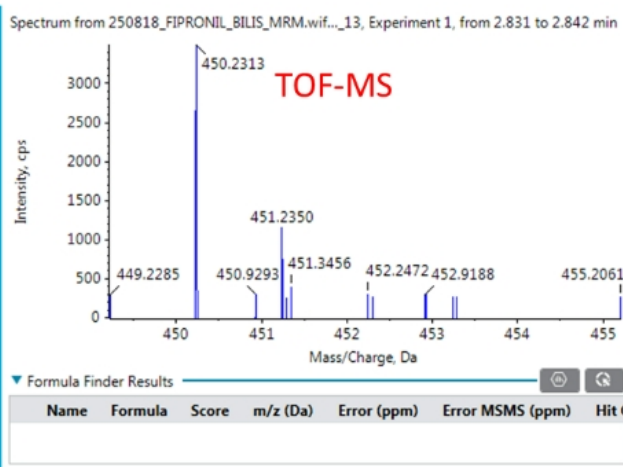
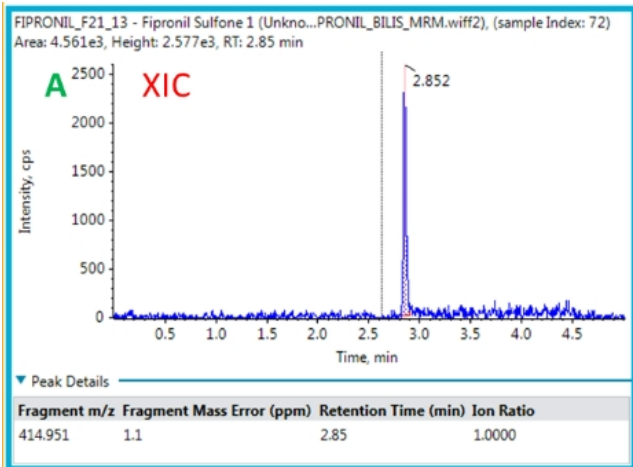
A)



B)







Electronic supplementary material (ESM)

Materials and Methods

Chemical analyses on bile

Fipronil reference standards (fipronil, fipronil sulfone, fipronil-desulfinyl, and fipronil-(pyrazole-¹³C₃, cyano-¹³C)) 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-¹³C₃, cyano-¹³C) 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 ($5(\text{TFA-Na})+\text{TFA}^-$, 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 °C 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 μ L ethyl acetate, 2 μ L 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 μ m film thickness. Injector temperature was 260 $^{\circ}$ C. The GC oven temperature program was as follows: 60 $^{\circ}$ C held for 1 min, ramped at 20 $^{\circ}$ C/min to 160 $^{\circ}$ C held for 1 min, then ramped at 3 $^{\circ}$ C/min to 200 $^{\circ}$ C held for 2 min, followed by 4 $^{\circ}$ C/min to 250 $^{\circ}$ C and held for 4 min. MSD transfer line was at 250 $^{\circ}$ C, and ion source was set at 200 $^{\circ}$ C. 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 °C and 16 °C) 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 quantified 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 \pm 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 °C				16 °C			
		t0	t7	t14	t21	t0	t7	t14	t21
Plasma	Glucose	11.04 \pm 1.42	19.04 \pm 2.60	15.23 \pm 1.91	21.23 \pm 2.22	11.86 \pm 1.46	19.19 \pm 1.99	15.66 \pm 0.93	15.58 \pm 1.32
	Lactate	5.27 \pm 0.55	6.36 \pm 1.11	6.50 \pm 0.45	7.81 \pm 0.88	5.83 \pm 0.41	7.93 \pm 0.71	6.60 \pm 0.44	7.55 \pm 0.54
	Osmolality	370.50 \pm 4.69	390.86 \pm 8.03	383.29 \pm 4.92	392.63 \pm 3.98	374.57 \pm 5.61	397.29 \pm 4.92	395.00 \pm 7.15	400.50 \pm 9.12
Mucus	Glucose	1.17 \pm 0.22	1.10 \pm 0.14	0.88 \pm 0.10	0.57 \pm 0.07	1.09 \pm 0.09	1.10 \pm 0.15	1.24 \pm 0.11	0.60 \pm 0.09
	Lactate	0.74 \pm 0.13	0.71 \pm 0.16	0.76 \pm 0.11	0.42 \pm 0.08	0.64 \pm 0.09	1.08 \pm 0.12	1.11 \pm 0.14	0.49 \pm 0.09
	Glucose/protein	6.15 \pm 1.67	6.39 \pm 0.98	3.31 \pm 0.47	5.99 \pm 1.42	4.02 \pm 0.61	4.97 \pm 0.75	4.64 \pm 0.64	4.11 \pm 0.54
	Lactate/protein	4.00 \pm 0.92	3.57 \pm 0.65	2.70 \pm 0.36	3.30 \pm 0.56	2.28 \pm 0.24	4.59 \pm 0.37	3.94 \pm 0.50	3.10 \pm 0.50
	Glucose/lactate	1.61 \pm 0.25	1.65 \pm 0.21	1.37 \pm 0.10	1.95 \pm 0.29	1.67 \pm 0.16	1.06 \pm 0.12	1.22 \pm 0.15	1.17 \pm 0.11

Table S2. Values of statistical parameters obtained when testing relationships between biomarkers and fish biological variables (SL: standard length, TW: total weight, 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 and 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	–	–	–	–	–	–	–
Liver - cytosol	LPO	0.286*	–	–	–	–	–	–
	GR	-0.336*	-0.494***	-0.299*	–	–	-0.333**	–
	t-GPX	–	–	–	–	–	–	–
	CAT	–	–	–	–	–	–	–
Liver -microsomes	GST	0.308*	0.423**	0.305*	–	–	0.320*	2.707**
	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*	–	–	–	–	–
Digestive tract - anterior	Glucose/lactate	–	–	–	–	–	–	–
	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 \pm 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 °C				16 °C			
		t0	t7	t14	t21	t0	t7	t14	t21
Plasma	Lysozyme	7.14 \pm 0.38	11.60 \pm 1.25	8.11 \pm 0.70	10.73 \pm 1.02	6.24 \pm 1.45	9.03 \pm 2.78	12.36 \pm 0.50	13.24 \pm 2.53
Muscle	LPO	2.99 \pm 0.74	3.03 \pm 0.46	5.57 \pm 1.37	4.12 \pm 0.72	3.14 \pm 1.06	6.21 \pm 1.09	5.30 \pm 0.70	5.41 \pm 1.88
Liver (S9)	LDH	15.11 \pm 0.90	14.85 \pm 1.11	16.25 \pm 1.17	16.23 \pm 1.00	15.48 \pm 1.48	14.58 \pm 1.42	15.61 \pm 1.37	15.49 \pm 1.38
	LPO	10.10 \pm 1.74	15.81 \pm 1.72	14.12 \pm 1.57	16.82 \pm 2.18	10.15 \pm 1.45	13.69 \pm 1.21	13.02 \pm 1.62	17.35 \pm 1.96
Liver (cytosol)	GR	7.48 \pm 1.02	6.03 \pm 0.86	6.85 \pm 0.68	7.98 \pm 0.84	7.39 \pm 0.63	7.20 \pm 0.94	5.56 \pm 1.05	7.84 \pm 1.17
	t-GPX	7.11 \pm 0.37	6.60 \pm 0.40	6.70 \pm 0.33	7.16 \pm 0.54	8.12 \pm 0.40	6.64 \pm 0.38	7.13 \pm 0.52	7.97 \pm 0.31
	CAT	103.68 \pm 12.47	90.70 \pm 8.68	92.85 \pm 11.35	63.84 \pm 10.27	96.70 \pm 7.19	108.92 \pm 8.75	111.12 \pm 6.28	76.88 \pm 11.33
	GST	36.94 \pm 1.73	39.49 \pm 1.51	42.83 \pm 3.52	43.26 \pm 2.71	39.44 \pm 2.62	36.45 \pm 2.43	48.27 \pm 2.52	51.05 \pm 5.80
Liver (microsomes)	EROD	22.04 \pm 2.09	18.75 \pm 2.98	15.48 \pm 1.38	15.33 \pm 1.31	25.35 \pm 3.86	24.36 \pm 4.37	17.01 \pm 3.23	18.14 \pm 2.56
	BROD	0.69 \pm 0.06	0.66 \pm 0.07	0.54 \pm 0.05	0.55 \pm 0.07	0.76 \pm 0.11	0.63 \pm 0.07	0.49 \pm 0.08	0.47 \pm 0.07
	MROD	4.70 \pm 0.32	3.74 \pm 0.51	2.80 \pm 0.34	2.34 \pm 0.18	5.00 \pm 0.71	4.17 \pm 0.64	2.91 \pm 0.60	3.40 \pm 0.78
	BFCOD	62.91 \pm 5.23	88.69 \pm 13.95	94.29 \pm 11.36	98.97 \pm 22.34	87.23 \pm 19.95	65.81 \pm 10.38	68.65 \pm 12.07	64.05 \pm 9.80
	CECOD	31.06 \pm 5.11	30.65 \pm 4.68	19.14 \pm 1.63	21.66 \pm 2.09	33.45 \pm 5.34	29.98 \pm 3.73	23.44 \pm 4.39	24.80 \pm 2.31
	ECOD	9.42 \pm 1.47	8.51 \pm 1.66	3.99 \pm 1.05	3.35 \pm 0.66	10.03 \pm 3.17	6.34 \pm 1.40	6.53 \pm 3.52	7.18 \pm 1.97
	UDPGT	0.78 \pm 0.09	0.76 \pm 0.07	0.77 \pm 0.14	0.78 \pm 0.08	0.81 \pm 0.13	0.75 \pm 0.09	0.77 \pm 0.08	0.82 \pm 0.08
	NADPH Cyt C reductase	19.42 \pm 0.98	19.06 \pm 1.08	22.14 \pm 1.57	22.98 \pm 2.24	18.04 \pm 1.49	17.07 \pm 1.22	18.99 \pm 1.11	18.94 \pm 1.44
	NADH Cyt C reductase	31.94 \pm 2.33	23.58 \pm 4.11	22.79 \pm 3.36	23.12 \pm 3.84	31.97 \pm 5.34	28.69 \pm 3.80	26.15 \pm 4.86	29.02 \pm 3.60
	NADH Ferricyanide reductase	1079 \pm 93	1145 \pm 111	1167 \pm 103	1095 \pm 66	1184 \pm 107	1006 \pm 95	1208 \pm 108	1184 \pm 75
Kidney	Na ⁺ /K ⁺ -ATPase	1.94 \pm 0.51	1.17 \pm 0.14	1.39 \pm 0.27	1.40 \pm 0.13	1.10 \pm 0.17	0.91 \pm 0.11	1.09 \pm 0.22	1.35 \pm 0.17

Table S4. Mean \pm 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 \pm 10.56	60.80 \pm 2.25	48.80 \pm 1.25	49.90 \pm 7.52	61.20 \pm 2.63	45.60 \pm 3.07
		Chymotrypsin	104.70 \pm 33.26	94.70 \pm 32.25	80.50 \pm 10.13	95.40 \pm 33.75	69.00 \pm 24.63	61.60 \pm 15.87
		Lipase	42.70 \pm 11.33	23.50 \pm 2.48	21.80 \pm 3.23	27.30 \pm 3.52	34.10 \pm 4.57	21.50 \pm 3.73
		Alpha-amylase	7.70 \pm 1.33	4.40 \pm 0.92	4.50 \pm 1.54	5.50 \pm 0.41	5.30 \pm 0.92	5.70 \pm 1.26
		Non-specific esterases	523.70 \pm 55.64	517.70 \pm 77.30	457.90 \pm 34.76	541.50 \pm 32.53	564.40 \pm 35.19	415.60 \pm 38.22
	Brush border	Alkaline-phosphatase	918.00 \pm 309.79	966.60 \pm 233.57	1,023.40 \pm 147.92	1,026.40 \pm 163.29	742.40 \pm 116.31	1,177.40 \pm 284.87
		Aminopeptidase	148.10 \pm 24.04	218.80 \pm 33.60	220.50 \pm 22.80	177.80 \pm 25.03	117.30 \pm 22.32	149.20 \pm 22.70
		Maltase	42.90 \pm 8.63	250.10 \pm 50.02	242.30 \pm 24.02	43.50 \pm 3.30	127.10 \pm 21.62	214.00 \pm 39.23
Digestive tract posterior	Pancreatic	Trypsin	57.10 \pm 12.98	47.70 \pm 2.55	47.80 \pm 1.78	64.40 \pm 10.03	52.10 \pm 3.38	45.70 \pm 3.22
		Chymotrypsin	117.30 \pm 39.39	231.40 \pm 84.12	176.10 \pm 59.25	340.30 \pm 142.84	180.20 \pm 85.15	332.30 \pm 145.94
		Lipase	30.00 \pm 5.69	30.80 \pm 3.94	30.40 \pm 6.06	27.30 \pm 8.46	28.60 \pm 3.71	24.90 \pm 3.82
		Alpha-amylase	3.10 \pm 0.44	4.90 \pm 0.79	9.70 \pm 2.48	3.40 \pm 0.40	5.80 \pm 1.15	6.20 \pm 1.52
		Non-specific esterases	587.70 \pm 56.37	477.20 \pm 43.85	519.70 \pm 27.89	481.00 \pm 12.44	553.70 \pm 58.99	447.40 \pm 43.69
	Brush border	Alkaline-phosphatase	626.70 \pm 193.56	826.20 \pm 175.18	749.70 \pm 97.03	776.90 \pm 95.46	540.80 \pm 109.19	745.00 \pm 176.76
		Aminopeptidase	97.20 \pm 6.81	159.30 \pm 21.09	178.90 \pm 27.34	154.20 \pm 19.76	93.50 \pm 11.08	104.70 \pm 15.78
		Maltase	26.20 \pm 2.92	158.20 \pm 23.95	150.50 \pm 25.26	32.80 \pm 2.44	93.60 \pm 11.88	116.30 \pm 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)	R²	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. Desulfinyl	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. Dallarés, P. Dourado, M. Blázquez and M. Solé: 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.