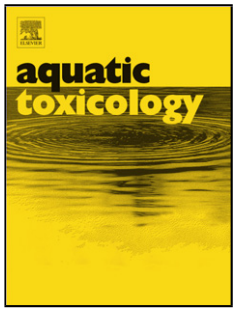
Journal Pre-proof



Multibiomarker approach to fipronil exposure in the fish *Dicentrarchus labrax* under two temperature regimes

Sara Dallarés, Priscila Dourado, Ignasi Sanahuja, Mikhail Solovyev, Enric Gisbert, Nicola Montemurro, Amparo Torreblanca, Mercedes Blázquez, Montserrat Solé

PII: S0166-445X(19)30775-1

DOI: https://doi.org/10.1016/j.aquatox.2019.105378

Reference: AQTOX 105378

To appear in: *Aquatic Toxicology*

Received Date: 20 September 2019

Revised Date: 26 November 2019

Accepted Date: 30 November 2019

Please cite this article as: Dallarés S, Dourado P, Sanahuja I, Solovyev M, Gisbert E, Montemurro N, Torreblanca A, Blázquez M, Solé M, Multibiomarker approach to fipronil exposure in the fish *Dicentrarchus labrax* under two temperature regimes, *Aquatic Toxicology* (2019), doi: https://doi.org/10.1016/j.aquatox.2019.105378

Multibiomarker approach to fipronil exposure in the fish *Dicentrarchus labrax* under two temperature regimes

Sara Dallarés[a], Priscila Dourado[b], Ignasi Sanahuja[c], Mikhail Solovyev[d,e], Enric Gisbert[f], Nicola Montemurro[g], Amparo Torreblanca[h], Mercedes Blázquez[a], Montserrat Solé[a,*]

[a] Institute of Marine Sciences (ICM-CSIC), Pg. Marítim de la Barceloneta 37–49, 08003 Barcelona, Spain

[b] Institute of Biosciences, Language and Exact Sciences of São José do Rio Preto, Paulist State University "Júlio de Mesquita Filho", Rua Cristóvão Colombo - de 1897/1898 ao fim, Jardim Nazareth 15054000 São José do Rio Preto, SP, Brasil

[c] Department of Cell Biology, Physiology and Immunology, Faculty of Biology, University of Barcelona, Avda. Diagonal 643, 08028 Barcelona, Spain

[d] Institute of Systematics and Ecology of Animals, Siberian Branch of Russian Academy of Sciences, Frunze st., 11, 630091 Novosibirsk, Russia

[e] Tomsk State University, 36 Lenin Ave, 634050 Tomsk, Russia

[f] Institute of Research and Technology Food and Agriculture (IRTA), Aquaculture Program, Ctra. Poble Nou, km 5.5, 43540 Sant Carles de la Ràpita, Tarragona, Spain

[g] Water and Soil Quality Research Group (IDAEA-CSIC), Department of Environmental Chemistry, C/Jordi Girona 18–26, 08034 Barcelona, Spain

[h] Department of Functional Biology and Physical Anthropology, University of València, C/Dr. Moliner 50, Burjassot 46100 Valencia, Spain

*Corresponding author: E-mail address: msole@icm.csic.es Tel: +34 932309500

Highlights

- Fipronil induces physiological alterations on European sea bass
- Stress markers were altered and oxidative-stress was induced
- Inhibition of phase I CYP activities and increase of phase II GST activity occurred
- A three-degree temperature increase did not enhance fipronil effects
- The metabolite fipronil-sulfone persisted in bile even after the depuration period

Abstract

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

inhibition of Cytochrome P450-related activities and an inhibition of phase II glutathione-S-transferase. Moreover, fipronil administration was able to reduce the hypo-osmoregulatory capability as shown by the increase of plasmatic osmolality and altered several digestive enzymes including trypsin, lipase, alpha amylase and maltase. Finally, analyses in bile and muscle confirmed the rapid clearance of fipronil but the persistence of the metabolite fipronil-sulfone in bile even after the 7-day depuration period. Altogether, the results reveal a notable impact of this compound on the physiological condition of the European sea bass. The results should be considered in future environmental risk assessment studies since fipronil could be hazardous to fish species, particularly those inhabiting estuarine ecosystems exposed to the discharge of agriculture runoffs where this pesticide is mainly used.

Keywords: fipronil, sea bass, biomarkers, CYP metabolism, oxidative stress, climate change.

1. Introduction

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

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

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

Human activities, including the input of pesticides into the environment, have been considered as the main cause for the present world climate change scenario (CC) (Hansen et al., 2006). Current consensus alerts that significant temperature increases, acidification and greater salinity fluctuations of marine water bodies will occur around the globe in the upcoming decades (IPCC, 2014). These changes can exert a direct impact on the physiology of marine poikilotherms (Makrinos and Bowden, 2016; Boltana et al., 2017; Navarro et al., 2019). On the other hand, indirect effects caused by CC in marine fish are still poorly known, especially those related to their potential interaction with foreign chemicals (Schiedek et al., 2007; Hooper et al., 2013). For instance, changes in physical conditions and chemical exposure can act synergistically magnifying the consequences of such exposures in aquatic organisms, since the former can imply changes in the availability and action of chemicals (Sokolova and Lannig, 2008; Jacquin et al., 2019). The Mediterranean region is especially sensitive to the alterations induced by CC due to its particular characteristics, such as small size, relatively shallow average water depth, oligotrophy and high biological diversity, among others (Calvo et al., 2011).

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

changes in water temperature are known to adversely affect a wide number of biological functions in this species including sex ratios, reproduction, growth, immune response, osmoregulatory capacity, xenobiotic biotransformation and antioxidant defences, among others, making the fish more vulnerable to additional stressors (Almeida et al., 2015; Samaras et al., 2018). Furthermore, metabolic alterations in muscle, liver and brain in response to a 4 ºC increase were enhanced after exposure to methylmercury in the European sea bass, raising the possibility for a synergistic effect between both stressors (Maulvault et al., 2017).

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

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

2. Material and Methods

2.1. Experimental design

Juvenile European sea bass (8 months old) were obtained from the Institute of Research and Technology Food and Agriculture (IRTA, Sant Carles de la Ràpita, Spain). Fish were transported and maintained at the Experimental Aquaria facilities (ZAE) of the Institute of Marine Sciences (ICM-CSIC, Barcelona, Spain). Prior to the experiment, fish were acclimated for a two-week period in a 2,500 L round fiberglass tank containing filtered sea-water (sterilized sand filter 50 μm) under natural conditions of temperature and with a water full-renovation rate of 24 times per day. Fish were fed daily *ad libitum* with commercial pellets (L-4 Optibass 2P, Skretting, Spain). After an initial two-week acclimation to lab conditions, fish were randomly assigned to four 600 L round fiberglass tanks (19–20 individuals per tank). Two of them were reared at natural water temperature (T ≈ 13 ºC) and the other two at 3 ºC above the natural temperature (T ≈ 16 ºC). The new experimental temperature was gradually attained at an increasing rate of 1 °C per day and fish were acclimated to these new conditions for two additional weeks. Then, fish were fed a diet containing Regent®800WG (80% fipronil) at a concentration of active ingredient of 10 mg fipronil/Kg feed, which was prepared following the alcohol evaporation method adapted for sea bass (Blázquez et al., 1995; Blanco et al., 2016). Briefly, a monolayer of pelleted dry feed was carefully sprayed with the insecticide dissolved in 15 ml ethanol and the solvent was allowed to evaporate completely at room temperature and kept stored at 4 ºC until used. Fish were sampled just before the start of the experimental diet (t0) and considered as control, and after 7 (t7), and 14 (t14) days of fipronil administration. At this point (t14), fipronil treatment finished and fish were fed with non-spiked commercial feed (depuration period) for an extra week completing 21 days from the start of the experiment (t21). Throughout the experiment, total feed consumption was quantified in each tank by initially weighing the amount before manual feeding and, when fish stopped feeding,

weighing the remaining amount; consumption was then calculated by weight difference. During the experiment, values (mean ± standard deviation) of physical water parameters were: Temperature = 13.37 ± 0.23 for the groups reared at natural temperature and 16.55 ± 0.44 ºC for those reared at +3 ºC. Other water parameters ranged as follows: dissolved O_2 = 6.66 ± 0.32 and 6.59 ± 0.47 mg/L (81% and 85% saturation, respectively); pH = 7.73 ± 0.35 and 7.64 ± 0.31; salinity = 37.78 ± 0.12 and 37.93 ± 0.27 psu, for the 13 ºC and 16 ºC groups, respectively. During the experiment, fish were reared under natural photoperiod corresponding to 10 h light:14 h dark.

2.2. Fish Sampling

Eight fish were sampled for each temperature regime (4 fish / replicate tank) just prior to the beginning of the exposure period (t0), after 7 (t7) and 14 (t14) days of fipronil administration, and after 7 days of depuration (t21). Fish were fasted for 48 h before each sampling time. Fish were anesthetized with 0.2 % 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO, USA), measured (standard length: SL) and weighed (total body weight: BW). Epidermal mucus was collected on sterile glass slides from the over-lateral line in caudal direction with especial care to avoid contamination with blood and/or urogenital and intestinal excretions (Fernandez-Alacid et al., 2018). Slides were gently wiped along both sides of the animal twice or three times, and mucus was carefully kept in a 1.5 mL sterile tube, snap frozen in liquid nitrogen and stored at -80ºC until use. About 1 ml of blood was withdrawn from the caudal vein using heparinized syringes and kept on ice until centrifugation. Fish were sacrificed by severing their spinal cord, eviscerated, weighed (EW) and the weight of liver, gonads and visceral fat recorded. Organs/tissues, including liver, bile, gonads, digestive tract, kidney and a

portion of axial muscle were collected and immediately frozen in liquid nitrogen and kept at –80 ºC for further analyses.

Muscle and bile samples were used for chemical analyses while plasma, skin mucus, liver, kidney, digestive tract and also muscle samples were used to assess a comprehensive set of biomarkers reflecting different aspects of sea bass physiology and metabolism (see sections below).

Fish were reared and sacrificed according to the Spanish regulations (RDL 53/2013), and the European Directive concerning the protection of vertebrates used for experimental and other scientific purposes (2010/63/EU). Procedures used were approved by the ethics committee of the Local Government of Catalonia and were given the reference FUE-2018-00813667. All steps were aimed to minimise animal suffering.

2.3. Tissue preparation for biochemical analyses

2.3.1. Plasma and skin mucus

Plasma was obtained by blood centrifugation (eppendorf 5417R model) at 3,000 ×g for 15 min at 4 ºC). Mucus was homogenized using a sterile Teflon implement and centrifuged at 14,000 ×g for 15 min at 4 ºC. Plasma and mucus supernatants were aliquoted and stored at –80 ºC for further metabolite and biochemical analyses.

2.3.2. Muscle

A portion of muscle (around 0.4 g) was homogenized in ice-cold buffer phosphate (50 mM pH 7.4) containing 1mM ethylenediaminetetraacetic acid (EDTA) in a 1:5 (w:v) ratio using a Polytron® homogeniser. Homogenates were centrifuged at 10,000 ×g for 30 min at 4 ºC to obtain the S10 fraction. The supernatant was aliquoted and stored at –80 ºC for further biochemical determinations.

2.3.3. Liver

About 1.5 g of each liver were homogenized in ice-cold buffer phosphate (100 mM pH 7.4) containing 150 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM phenanthroline, 0.1 mg/mL trypsin inhibitor and 1 mM EDTA in a 1:4 (w:v) ratio using a Polytron® blender. Homogenates were centrifuged at 10,000 ×g for 30 min at 4 °C to obtain the S10 fraction, of which 1 mL was withdrawn while the rest was further homogenised at 100,000 ×g for 60 min at 4 °C to obtain microsomal and cytosolic fractions. Microsomal pellets were dissolved in the above-described homogenization buffer, also containing 20 % glycerol in a 2:1 (w:v) ratio (Crespo and Solé, 2016). S10, microsomal and cytosol fractions were aliquoted and stored at –80 ºC for further biochemical determinations.

2.3.4. Kidney

About 0.05–0.1 g of each individual kidney were homogenised in ice-cold buffer (pH 7.3) containing 150 mM reagent-grade sucrose, 50 mM imidazole and 10 mM Na_2EDTA in a 1:15 (w:v) ratio using a Polytron® blender. Homogenates were centrifuged at 5,000 ×g for 2 min at 4 ºC. The resulting supernatant was aliquoted and stored at –80 ºC for osmoregulation and enzymatic measures. A more detailed description is given in González-Mira et al. (2018).

2.3.5. Digestive tract

The intestines were divided into anterior and posterior regions of equal length and in each of them, pancreatic (trypsin, chymotrypsin, bile salt activated lipase and alpha-amylase) and intestinal brush border (BB) enzymatic activities (alkaline phosphatase,

aminopeptidase N and maltase) were quantified. Further methodological details can be found elsewhere for pancreatic enzymes (Gisbert et al. 2009) and intestinal enzymes (Gisbert et al. 2018). The activity of non-specific esterases was also determined in the pancreatic fraction. Intestines from t7 group were discarded from the study because in this case fish were fasted only for 24 h, as opposed to 48 h in the other groups, something that could affect the activity of digestive enzymes.

2.4. Biochemical analyses

All reactions were carried out in triplicate at 25 ºC, except for CYPs, UDPGT and digestive enzymes determinations, which were measured at 30 ºC on a Tecan™Infinite M200 spectrophotometer.

2.4.1. Plasmatic and skin mucus metabolites and lysozyme determination

Glucose and lactate content in plasma and skin mucus (expressed as µg/mL) were determined by enzymatic colorimetric kit tests GOD-POD glucose (Ref: 41011) and LO-POD lactate (Ref: 1001330), from SPINREACT® (Spain), according to the methodology described in Fernández-Alacid et al. (2018).

Plasmatic ammonia (expressed as µmol/L) was analyzed using a commercial kit by SPINREACT®, and (Ref: 1001410).

Lysozyme activity in plasma (expressed as units (U)/ mg of total plasmatic protein) was measured according to the turbidimetric method described by Parry et al. (1965) with some modifications. Briefly, 100 µL of plasma diluted in a 1:2 ratio with 10 mM PBS pH 6.2 were placed in flat-bottomed 96-well plates. To each well, 100 µL of freeze-dried *Micrococcus lysodeikticus* (0.3 mg/ml, Sigma) were added as lysozyme substrate. The absorbance (λ = 450 nm) was measured at the beginning and after 15 min. Units of

lysozyme present in plasma were obtained from a standard curve built with chicken egg white lysozyme (HEWL, Sigma).

2.4.2. Anaerobic metabolism

Lactate dehydrogenase (LDH) activity was measured in the S10 fraction of the liver following adaptation of the Vassault (1983) method using NADH (200 µM) and pyruvate (1 mM) as final well concentrations. Reading was done at $\lambda = 340$ nm for 5 min. LDH activity was expressed as nmol/min/mg total protein.

2.4.3. Oxidative-stress parameters

Activities of the antioxidant enzymes catalase (CAT), total glutathione peroxidase (GPX) and glutathione reductase (GR) were determined in the liver cytosolic fraction. CAT activity was measured as a decrease in absorbance at $\lambda = 240$ nm using H_2O_2 (50 mM) as substrate; GPX and GR used cumene hydroperoxide (CHP, 0.625 mM) and oxidized glutathione (GSSG, 0.9 mM) as respective substrates and NADPH as cofactor in both assays at $\lambda = 340$ nm. Lipid peroxidation levels (LPO) were quantified in muscle and in S10 liver fraction using a colorimetric method with 1-methyl-2-phenylindole. Quantification, with respect to the standard solution 1,1,3,3-tetramethoxypropane, was made at $\lambda = 586$ nm. CAT activity was expressed as µmol/min/mg total protein and GR and GPX activities as nmol/min/mg total protein and LPO levels as nmol MDA (malondialdehyde)/g wet weight.

2.4.4. Conjugation enzymes

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

λ = 340 nm. Uridine diphosphate glucuronyltransferase (UDPGT) activity was measured in liver microsomes according to the method of Collier et al. (2000) using methyl umbelliferone (MU, 0.1 mM) as substrate in the fluorometric mode (EX/EM 355/460). Both activities were expressed as nmol/min/mg total protein.

2.4.5. CYP components and reductases

Catalytic activities of hepatic CYPs were determined in the microsomal liver fraction using six fluorometric substrates: 7-ethoxyresorufin (ER), 7-benzyloxyresorufin (BR), 7-methoxyresorufin (MR), 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 3-cyano-7-ethoxycoumarin (CEC) and 7-ethoxycoumarin (EC). Assay conditions were based on the method by Solé et al. (2012). Briefly, microsomes (10 µL) were incubated for 10 min at 30 ºC and the metabolite formed was recorded at its specific wavelength (Smith and Wilson, 2010). A calibration curve for each specific metabolite was done (range 0–160 nM). CYPs assays were run in 100 mM phosphate buffer pH 7.4, except for ECOD determination, which was done in 100 mM Tris buffer pH 7.4. Activities were expressed in pmol/min/mg total protein.

Microsomal reductases, NAD(P)H- cytochrome c reductases and NADH- ferricyanide reductase activities, were measured by the increase in absorbance at λ = 550 nm and the decrease in absorbance at λ = 420 nm, respectively (Solé and Livingstone, 2005). Assay conditions were: 50 mM Tris-HCl buffer pH 7.6, 1 mM KCN, 0.26 mM NAD(P)H, and 60 µM cytochrome c or 0.2 mM potassium ferricyanide. Sample volumes were: 10 µL microsomal fraction for NADPH- and 15 µL for NADH-dependent reductases. Results are expressed in nmol/min/mg total protein.

2.4.6. Osmoregulation

Activity of Na^+/K^+-ATPase was determined in the head kidney of fish following Zaugg (1982) method with modifications (González-Mira et al., 2018). Sample absorbance was measured at λ = 750 nm after 30 min incubation. Na^+/K^+-ATPase activities were expressed as µmol ATP hydrolysed/mg total protein/hour. Plasmatic osmolality was measured with the aid of a Fiske® 210 Micro-Sample Osmometer using 20 µL plasma and expressed in mosm/Kg H_2O.

2.4.7. Digestive enzymes

The methods used for enzyme quantification are briefly described as follows: trypsin and chymotrypsin activities, the two main pancreatic alkaline proteases, were assayed using, respectively, N-benzoyl-DL-arginine p-nitroanilide (BAPNA) (Holm et al., 1988) and Succinyl-L-Ala-Ala-Pro-L-Phenylalanine p-nitroanilide (SAAPNA) (Erlanger et al., 1961). Alpha-amylase activity was estimated using 2-chloro-p-nitrophenyl-α-D-maltotrioside as substrate (Lorentz et al., 1999). The activities of bile-salt-activated lipase and non-specific esterases were measured using p-nitrophenyl myristate (Iijima et al., 1998) and p-nitrophenyl acetate (Hosokawa and Satoh, 2005) as respective substrates. The activity of the alkaline phosphatase was determined using 4-nitrophenylphosphate (Bessey et al., 1946), aminopeptidase N activity was determined using L-leucine p-nitroanilide (Maroux et al., 1973) and maltase activity was determined using d(+)-maltose (Dahkqvist, 1970) as substrates. All enzymatic activities were expressed as specific units (mU/mg total protein).

2.4.8. Protein determination

Total protein content of all samples was determined by the Bradford method (1976) using the Bio-Rad Protein Assay reagent and bovine serum albumin (BSA; 0.05-1 mg/mL) as standard. The absorbance was read at $\lambda = 595$ nm.

2.5. Chemical analyses

2.5.1. Bile

Bile glands from group t7 were mostly empty (as described in digestive enzyme measures), and could not be used for chemical analysis. A more detailed methodology (adapted from Aceña et al. (2017)), as well as reference standards and solvent solutions characteristics and a description for fipronil and its metabolites quantification, is reported as electronic supplementary material (ESM).

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).

2.5.2. Muscle

Analysis of fipronil in muscle was based on the use of the commercial brand Regent®800WG (80% fipronil) as standard and following the solid phase QuEChERS extraction method and gas chromatography-electron impact (GC-EI) detection at the Scientific and Technological Centres of the University of Barcelona (CCiTUB) that holds the quality standard ISO 9001:2015. A more detailed description of the analytical procedure is provided as ESM.

2.6. Data analyses

Fish hepatosomatic index (HSI) and gonadosomatic index (GSI) were calculated as (liver weight/BW) × 100 and (gonad weight/BW) × 100, respectively. Fish condition was assessed by Fulton's condition factor (CF), calculated as (BW/TL^3) × 100.

Daily feed intake was measured per tank and food consumption calculated in relation to the total number of fish per tank. For each mucus sample, the ratios of glucose/lactate, glucose/protein and lactate/protein were calculated.

A detailed explanation of statistical procedures is provided as ESM. In short, relationships among fish biological and some biochemical variables and their interactions were tested by Pearson' or Spearman' rank correlations (continuous variables) and by Student's t-tests or Mann-Whitney U-tests (sex-related differences). For digestive enzymes, differences between anterior and posterior parts of the digestive tract were tested using Wilcoxon pairwise tests with repeated measurements. Possible effects of treatment duration and temperature were tested by general or generalized linear models (GLMs/GZMs) followed by Student's t-test/Mann-Whitney U-test and one-way ANOVA/Kruskal-Wallis tests. A permutation multivariate analysis (PERMANOVA) and a principal component analysis (PCA) were also carried out in order to assess a global biochemical response to temperature and treatment duration In all cases, significant differences were set at $p < 0.05$.

3. Results

3.1. Biometric parameters and general condition indices

Mean values for fish biometric data and general condition indices were fairly uniform across experimental groups (Table 1). Most individuals were immature, as evidenced by low GSI values in both sexes. Sex ratios were generally skewed, with more females

than males in most groups. Significant increases of SL and BW with time were observed (χ^2 = 10.589, p = 0.014 and χ^2 = 8.613, p = 0.035, respectively).

Direct correlations among biometric data and condition indices were found in most cases, with visceral fat weight showing positive associations with all other biological variables (r_p = 0.299–0.692, p = 0.018 – < 0.001), in a similar way as BW (only non-significant correlation to HSI) (r_p = 0.268–938, p = 0.032 – < 0.001). SL and GSI were also positively correlated (r_p = 0.483, p < 0.001), as well as CF and HSI (r_p = 0.457, p < 0.001). Body weight, GSI and visceral fat weight displayed higher values in females than in males (t = 2.690–6.252, p = 0.012– < 0.001).

3.2. Feed consumption

Fish feeding rate was similar at the two experimental temperatures: 0.54 ± 0.20 and 0.57 ± 0.22 g/fish at 13 ºC and 16 ºC, respectively, and significantly increased over time ($F_{(3, 24)}$ = 23.891, p < 0.001) (Table 1).

3.3. Biochemical analyses

3.3.1. Plasmatic and skin mucus analyses

Mean levels of plasmatic glucose ranged between 110.40 ± 14.20 and 212.30 ± 22.20 mg/dL and lactate between 52.70 ± 5.50 and 79.30 ± 7.10 mg/dL. In mucus these values were much lower with glucose ranging between 0.57 ± 0.07 and 1.24 ± 0.11 mg/dL and lactate between 0.42 ± 0.08 and 1.11 ± 0.14 mg/dL, (Table S1). Some statistical correlations of the different parameters studied in plasma and skin mucus and most fish biometrics and condition indices are detailed in Table S2.

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

7.098, $p < 0.001$ and $F_{(3, 53)} = 4.226$, $p = 0.009$, respectively) (Fig. 1A, B). Moreover, a decrease of these biomarkers during the depuration period was observed in skin mucus ($F_{(3, 59)} = 10.196$, $p < 0.001$ and $F_{(3, 59)} = 6.338$, $p = 0.001$, respectively) (Fig. 1C, D). Glucose/protein and lactate/protein ratios (mean values 3.31 ± 0.47 – 6.39 ± 0.98 µg/mg and 2.28 ± 0.24 – 4.59 ± 0.37 µg/mg, respectively) in skin mucus were not affected by temperature or fipronil exposure. However, glucose/lactate ratio (1.06 ± 0.12 – 1.95 ± 0.29 µg/mg) was higher at 13 ºC than at 16 ºC ($t = 2.733$, $p = 0.008$) (Table S1). A significant interaction between exposure time and CF was found for lactate content in mucus ($F_{(3, 54)} = 6.113$, $p = 0.001$). Strong positive correlations were detected among protein, glucose and lactate both in plasma ($r_p = 0.411–0.632$, $p < 0.001$) and skin mucus ($r_p = 0.434–0.706$, $p < 0.001$).

Plasmatic lysozyme activity ranged between 7.98 ± 2.13 and 13.24 ± 2.53 U/mg total protein (Table S3) and was not affected by temperature or fipronil exposure time.

3.3.2. Anaerobic metabolism

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

3.3.3. Oxidative-stress parameters

Antioxidant enzymes GR (5.56 ± 1.05 – 7.98 ± 0.84 nmol/min/mg total protein), GPX (6.60 ± 0.40 – 8.12 ± 0.40 nmol/min/mg total protein) and CAT (63.84 ± 10.27 – 111.12 ± 68 µmol/min/mg total protein) (Table S3) were not affected by temperature, and only CAT responded to fipronil exposure, decreasing after the depuration period (t21) ($F_{(3, 60)}$

= 4.792, p = 0.005) (Fig. 2A). Regarding fish biological variables, only some negative associations were observed between GR and some biological traits (Table S2).

Mean LPO levels ranged between 2.99 ± 0.74 and 6.21 ± 1.09 nmol MDA/g ww in muscle and between 10.10 ± 1.74 and 17.35 ± 1.96 nmol MDA/g ww in the S10 liver fraction (Table S3). In both cases, no effect of temperature was detected but a significant increase in MDA equivalents during fipronil exposure was found in liver ($F_{(3, 60)}$ = 7.436, p < 0.001) (Fig. 2B).

Correlations among oxidative stress-related biomarkers are shown in Table 2, revealing a similar trend for GR and GPX activities, while GR scaled negatively with CAT activity and LPO levels in liver.

3.3.4. Conjugation enzymes

Mean GST activity values ranged between 36.45 ± 2.43 and 51.05 ± 5.80 nmol/min/mg total protein, and UDPGT between 0.75 ± 0.09 and 0.82 ± 0.08 nmol/min/mg total protein (Table S3). Some positive correlations were detected between conjugation enzymes and biological variables, and GST activity was higher in females (Table S2). Both enzymes were unaffected by temperature and GST activity was significantly enhanced with increasing fipronil exposure time ($F_{(3, 60)}$ = 4.245, p = 0.009) (Fig. 2C). GST activity displayed positive correlations with LPO in muscle and liver and was negatively related to GR activity, while UDPGT activity was positively correlated with CAT activity (Table 2).

3.3.5. CYP components and reductases

EROD (mean activity = 15.33 ± 1.31 – 25.35 ± 3.86 pmol/min/mg total protein), BROD (0.47 ± 0.07 – 0.76 ± 0.11 pmol/min/mg total protein), MROD (2.34 ± 0.18 – 5.00 ±

0.71 pmol/min/mg total protein), CECOD (19.14 ± 1.63 – 33.45 ± 5.34 pmol/min/mg total protein) and ECOD (3.35 ± 0.66 – 9.42 ± 1.47 pmol/min/mg total protein) activities (Table S3) displayed negative correlations with fish HSI, as well as negative associations between MROD and visceral fat weight and between ECOD and CF (Table S2). These same formerly mentioned activities were unaffected by temperature but significantly decreased after fipronil exposure ($F_{(3, 60)}$ = 3.046, p = 0.036; $F_{(3, 60)}$ = 4.327, p = 0.008; $F_{(3, 60)}$ = 7.743, p < 0.001, $F_{(3, 60)}$ = 3.795, p = 0.015 and $F_{(3, 47)}$ = 3.211, p = 0.031, respectively) (Fig. 2D, E). By contrast, BFCOD activity (62.91 ± 5.23 – 98.97 ± 22.34 pmol/min/mg total protein) was not affected by temperature of fipronil exposure (Table S3). Strong positive correlations were found among most CYPs and between them and the oxidative-stress markers GR and GPX (Table 2). By contrast, negative associations were observed between GST and MROD and between UDPGT and most CYPs (Table 2).

In relation to CYP-dependent reductases (expressed in nmol/min/mg total protein): NAD(P)H Cyt c reductase (17.07 ± 1.22 – 22.98 ± 2.24) and NADH-Cyt c (22.79 ± 3.36 – 31.97 ± 5.34) and ferrycyanide reductases (1006 ± 95 – 1208 ± 108) (Table S3), NADPH cyt c reductase displayed higher activity at the lowest temperature (t = 2.575, p = 0.012) (Fig. 2F) and a positive weak correlation with fish visceral fat weight, and of NADH Cyt c reductase, that was negatively associated to fish GSI (Table S2).

3.3.6. Osmoregulation

Kidney Na^+/K^+-ATPase mean activity ranged from 0.91 ± 0.11 to 1.94 ± 0.51 µmoles of ATP hydrolysed/mg total protein/hour (Table S3), osmolality from 370.50 ± 4.69 to 400.50 ± 9.12 mosm/Kg H_2O and ammonia from 153.10 ± 9.46 to 333.62 ± 59.29 µmol/L (Table S1). While Na^+/K^+-ATPase activity and ammonia content did not

significantly vary as a function of temperature or experimental time, osmolality showed a significant increase after fipronil exposure ($F_{(3, 57)}$ = 6.729, p = 0.001).

3.3.7. Digestive enzymes

Significant differences in activity between anterior and posterior intestine sections were detected for the pancreatic enzymes trypsin (t14 at 13 °C; W = 36, p = 0.012) and chymotrypsin (t21 at 16 °C; W = 27, p = 0.038) and the BB enzymes alkaline phosphatase (t21 at 13 and 16 ºC; W = 27, p = 0.039 and W = 28, p = 0.023, respectively), aminopeptidase N (t21 at 13 and 16 ºC; W = 33, p = 0.043 and W = 35, p = 0.019, respectively) and maltase (t0 at 16 °C and t21 at 13 and 16 ºC; W = 21–36, p = 0.012–0.046). In light of these results, data from digestive enzymes for anterior and posterior intestine regions were treated separately.

Regarding pancreatic enzymes (in mU/mg total protein) in anterior intestine: trypsin (45.60 ± 3.07 – 61.20 ± 2.63), chymotrypsin (61.60 ± 15.87 – 104.70 ± 33.26), bile salt-activated lipase (21.50 ± 3.73 – 42.70 ± 11.33), alpha-amylase (4.40 ± 0.92 – 10.50 ± 3.01) and non-specific esterases (415.60 ± 38.22 – 564.40 ± 35.19) (Table S4), lower activities were observed for trypsin before fipronil exposure (t0) and after depuration period (t21) ($F_{(2, 41)}$ = 5.193, p = 0.01) (Fig. 2G), and for lipase and alpha-amylase over time ($F_{(2, 44)}$ = 3.435, p = 0.042 and $F_{(2, 45)}$ = 3.708, p = 0.033, respectively). For posterior intestine: trypsin (45.70 ± 3.22 – 64.40 ± 10.03), chymotrypsin (117.30 ± 39.39 – 428.50 ± 158.87), lipase (24.90 ± 3.82 – 30.80 ± 3.94), alpha-amylase (3.10 ± 0.44 – 9.70 ± 2.48) and non-specific esterases (447.40 ± 43.69 – 587.70 ± 56.37) no significant trends were detected. In relation to intestinal BB enzymes (in mU/mg total protein) in anterior intestine: alkaline phosphatase (742.40 ± 116.31 – 1,177.40 ± 284.87), aminopeptidase N (117.30 ± 22.32 – 220.50 ± 22.80) and maltase (42.90 ±

8.63 – 250.10 ± 50.02), higher activities were found for aminopeptidase N at 13 ºC (t = 2.310, p = 0.026) and for maltase with time exposure ($F_{(2, 45)}$ = 34.975, p < 0.001) (Fig. 2H). Among the enzymes assessed in posterior intestine: alkaline phosphatase (540.80 ± 109.19 – 826.20 ± 175.18), aminopeptidase N (93.50 ± 11.08 – 178.90 ± 27.34) and maltase (26.20 ± 2.92 – 158.20 ± 23.95), only maltase activity increased over time ($F_{(2, 45)}$ = 28.828, p < 0.001).

Regarding to association of digestive enzymes with fish biometric variables and general condition indices, few significant associations were found (Table S2) while mostly positive correlations were found among intestinal enzymes (Tables S5 and S6).

3.4. Chemical analyses on bile and muscle

Concentrations of fipronil and fipronil-sulfone in bile and in muscle (only fipronil) of the different experimental groups are shown in Table 3. In addition, a more detailed report on chemical results is provided as ESM.

3.5. Multivariate analyses

PERMANOVA analyses showed no effect of temperature but a significant influence of fipronil on fish general biochemical profile (*Pseudo-*$F_{(3, 60)}$ = 2.790, $p_{(perm)}$ = 0.0001; 9876 unique permutations, all pairwise comparisons significant except those comparing t7 and t14, and t14 and t21).

Two-dimensional PCA plots represented 56.1 % of total variance on the first two components, and 51.9 % of the total variance on the first and third components (Figure 3). These results suggest a differentiation according to the exposure time to fipronil along the first axis, with samples corresponding to unexposed fish (t0) (Fig. 3A, right part of the plot) clearly separated from the rest (Fig. 3A, left part of the plot), and

according to temperature along the third axis (Fig. 3B). Pearson correlations indicated associations between some biochemical markers and fish groups, namely between most CYP-related activities and unexposed fish (t0), between LPO levels, plasmatic metabolites and osmolality and fish from t7 and t14, and between LDH, NADPH-Cyt c reductase and GST and fish from t14 and t21.

4. Discussion

The present study reports, for the first time, the effects of dietary fipronil exposure on several physiological parameters of the European sea bass. The use of a comprehensive set of biomarkers encompassing different physiological and detoxification processes allows for the assessment of the effects of this pesticide on the health and general condition of an economically important cultured fish species. Moreover, the combined effects of fipronil and a 3 ºC temperature increase (as predicted for the NW Mediterranean region by the end of this century) constitute a novel approach to assess the consequences of CC for the harmful effects of this chemical in this commercial fish species widely used for human consumption.

Morphometric markers and condition indices remained unchanged regardless of fipronil exposure or of the rearing temperature, suggesting that the 3 ºC temperature variation, fipronil concentration and/or the time of exposure assessed were below threshold limits to affect them. The observed increase in feeding rate over time regardless of the temperature likely accounts for higher feed consumption as fish increased in size, and not to fipronil exposure, since no changes occurred during depuration.

Despite of the wide array of biochemical markers assessed in this study, the use of multivariate tools helped to infer some general patterns in their response to the different experimental conditions. It appears that temperature induced changes in some metabolic

parameters as shown by the segregation along the third PCA axis of fish reared at 13 ºC and 16 ºC but the PERMANOVA analyses indicated that the temperature-induced metabolic changes observed were not that clear. An integrated multi-biomarker response to fipronil exposure, according to both multivariate analyses, showed that the most prominent changes took place between unexposed (t0) and exposed (t7, t14 and t21) fish. Notably, biochemical patterns after depuration (t21) clustered with those for t14 fish both in PERMANOVA analysis and PCA plots, rather than with non-exposed groups (t0) suggesting that the 7-days depuration period was too short to allow for the full recovery of pre-exposure levels.

The choice of a modest temperature increase, 3 ºC with respect to the group reared at natural temperature, was considered as environmentally realistic under the IPCC forecasts by year 2100, although sharper increases may occur in estuarine and coastal ecosystems (IPCC, 2014) inhabited by European sea bass, particularly in their juvenile stage. The generalized lack of effects of this temperature increase on the assessed biomarkers contrasts with other studies performed on juveniles of this species, which reported behavioural, physiological and biochemical changes although under warmer conditions that could account for the different results (Vinagre et al., 2012; Almeida et al., 2015). Furthermore, a synergic effect between warmer conditions and fipronil exposure did not occur in the present study. However, one must keep into account that a more realistic simulation of CC conditions, including alterations of other abiotic variables (e.g. salinity, pH), could yield a different outcome.

In the present study, several parameters in plasma and mucus were included as potential non-lethal indicators of fish stress condition. Glucose and lactate increases in plasma could be a result of the mobilisation of energetic resources induced by higher metabolic demands, especially during the depuration period. In turn, the concomitant drop in

mucus could be explained by the need to spare energy when energetic demands increase, as described in gilthead sea bream during a 2-weeks starvation period (Fernández-Alacid et al., 2018) or under chronic cold temperature conditions (Sanahuja et al., 2019). Thus, plasma and mucus metabolite levels do not necessarily match under chronic or sustained stress conditions (several days-weeks), as it is observed in the present study and contrary to what has been reported under acute stress (hours) (Fernández-Alacid et al., 2019).

In fish, fipronil metabolism takes place by oxidation and reduction reactions catalysed by cytochrome P450-related enzymes (CYPs), which generate different fipronil metabolites (mainly fipronil sulfone) (Konwick et al., 2006; Wang et al., 2016; Li et al., 2018). These metabolites can be even more toxic to insects, mammals, aquatic organisms and birds than the parental compound (Leghait et al., 2009; Tavares et al., 2015). In the present study, this important phase I metabolic pathway was assessed by using several fluorometric substrates indicative of several CYP isoforms (Smith and Wilson, 2010; Solé et al., 2014) and the general electron donors NAD(P)H Cyt c and NADH ferrycyanide reductases. The responses of CYP1A- and CYP2B-related activities (EROD, BROD, MROD, ECOD and CECOD) showed a similar trend, markedly decreasing after fipronil administration, suggesting certain overlapping substrate specificity, whereas CYP3A4-associated BFCOD activity displayed the opposite trend. The effects of fipronil on CYP-related activities is controversial; for instance, *in vitro* studies with human hepatocytes (Das et al., 2006) suggested an increase in CYP1A1-related activity at low concentrations (1 µM), and a decrease at higher ones (10 and 25 µM). In the present study, ECOD activity was clearly inhibited after 14 days of fipronil exposure and remained low even at the end of the depuration period. This CYP-related activity was the only one responding to waterborne fipronil

exposure in the zebrafish, *Danio rerio*, showing a dose-dependent induction in several tissues 24h after exposure (Wu et al., 2014). A recent study in the Caspian kutum fish, *Rutilus kutum*, showed a strong correlation between *cyp1a* gene expression and different antioxidant responses in several tissues, including liver (Ardeshir et al., 2018). The study suggested that the increase of *cyp1a* gene expression after intraperitoneal (IP) fipronil injection could be due to structural similarities between this compound and some aryl hydrocarbon receptor agonists, such as halogenated hydrocarbons (Ardeshir et al., 2018). In fact, fipronil transformation into more toxic metabolites fipronil-sulfide and fipronil-sulfone is linked to oxidative stress (Wang et al., 2016). This is supported by the present results, since CAT, the antioxidant enzyme, was inhibited after the longest exposure to fipronil, supporting the notion that the production of oxyradicals may overwhelm the protective capacity of this enzyme (Regoli and Giuliani, 2014). Moreover, the increase of LPO levels in liver and muscle clearly confirmed a scenario of oxidative damage to cell membrane lipids, most likely due to ROS generation as previously suggested (Wang et al., 2016). In addition to the role of GST catalysing the conjugation of glutathione with xenobiotics for detoxification purposes, other GST isoforms appear implicated in the reduction of lipid hydroperoxides produced by ROS (Regoli and Giuliani, 2014). Present data point at this possibility, as suggested by the progressive increase in GST activity after fipronil exposure. Nonetheless, this increase in antioxidant protection, as indicated by the positive correlations between GST activity and LPO levels in liver and muscle was not enough to prevent the occurrence of oxidised lipids even after depuration. Concordantly, several studies performed in fish also reported the occurrence of oxidative-stress after fipronil administration, either waterborne exposure (Clasen et al., 2012; Menezes et al., 2016; Ghazanfar et al., 2018), after IP injection (Ardeshir et al., 2017a) and even considering a combination of

waterborne exposure and IP injection (Ardeshir et al., 2017b). Thus, induction of oxidative stress is a well-accepted consequence of fipronil exposure.

Since the European sea bass is a euryhaline fish that inhabits waters with broad salinity gradients during its life cycle, osmoregulation constitutes a key physiological process worth to be considered. In the present study, two osmoregulation-related parameters were assessed. A significant increase in plasmatic osmolality evidenced a reduced capability of hypo-osmoregulation in seawater after fipronil administration. However, another parameter also indicative of osmoregulation capacity such as kidney Na^+/K^+ ATPase activity was not affected. At this stage, we can only speculate that another response on this parameter might have been obtained if it had been measured in gills instead of kidney, since this marker seems to display a tissue-dependent pattern of activity (Vargas-Chacoff et al., 2009). Consequences for an osmoregulatory imbalance after fipronil exposure are particularly significant in this species, given that during early life stages it inhabits estuarine and freshwater ecosystems potentially subjected to waste water discharges from nearby agricultural areas where the insecticide may be used.

In the present study, the consequences of fipronil exposure were also evaluated in the digestive system because of the high importance of growth and energy assimilation in this cultured fish species. The effects of temperature and fipronil on the activity of the main digestive pancreatic and BB enzymes were evaluated in proximal and distal intestinal parts since fish intestine is characterized by proximo-distal gradients of hydrolases (Xiong et al., 2011; Izvekova et al., 2013). In the present study, the decrease on lipase activity in bile might be due to the presence of fipronil-derived compounds in bile, such as fipronil sulfone, that could have impaired lipase activity. Similarly, the pyretroid insecticide deltamethrin, used in combination with fipronil (Jiang et al., 2014), has been shown to inhibit lipase activity in several fish species (Simon et al., 1999;

Gunes and Yerli, 2011). Regarding the glucosidases alpha-amylase and maltase, suppression of the activity of the former has been reported in several fish species exposed to different pollutants, and mostly explained by a reduction in substrate affinity (Filippov et al., 2013). The opposite trend was observed for maltase activity in anterior and posterior intestine regions after fipronil exposure. This is in line with the random effects of toxics on glucosidases ranging from inhibition to stimulation depending on toxicant concentration, its interaction with other chemicals and exposure time (Filippov and Golovanova, 2012; Filippov et al., 2013). Therefore, the response of glucosidases to fipronil (whose effects on digestive enzymes have never been addressed before) needs to be further investigated before more consistent conclusions could be drawn. Similarly, the effect of organic pollutants on proteolytic activity (i.e. trypsin) seems inconsistent; while it significantly increased in the present study after fipronil administration, it decreased in roach (*Rutilus rutilus*) exposed to polychlorinated biphenyls (PCBs) (Golovanova et al., 2011) and it was unaffected by naphthalene (a polycyclic aromatic hydrocarbon) in Mozambique tilapia, *Oreochromis mossambicus*, (Kuz'mina et al., 1999). Since exposure to fipronil did not result in a decrease of BB enzymes, as indicative of harm to enterocytes integrity (Lalles, 2010), it seems that no damage occurred to intestinal epithelium at the tested concentration.

Chemical analyses in muscle and bile confirmed intake and clearance of fipronil during the exposure period as well as a bioaccumulation trend over time and a depuration after withdraw from diet. Higher levels of the metabolite fipronil-sulfone than those of the parental fipronil in fish bile at t14 and t21 confirmed a metabolisation of the insecticide within a few days. Notably, in rainbow trout, *Oncorhynchus mykiss* (Konwick et al., 2006) and Nile tilapia, *Oreochromis niloticus*, (Li et al., 2018), fipronil-sulfone was detected as soon as one day after exposure to the parent compound, indicating its rapid

biotransformation. In the present study, the parent compound fipronil was not detected in the muscle and very low concentrations were quantified in bile at the end of the 7-day depuration period (t21). This is in agreement with the rapid elimination reported by Konwick et al. (2006), who could not detect the pesticide in trout muscle 4 days after the end of the exposure. In contrast, fipronil-sulfone persisted in bile after the depuration period (7 days after the end of fipronil administration) at fairly high concentrations, which may be related to its affinity towards organic carbon supporting the view that fatty organs, such as liver, can act as a reservoir for fipronil residues (Li et al., 2018; Qu et al., 2018). Indeed, fipronil-sulfone is considered to be more toxic to aquatic species than fipronil itself (EPA, 1996). This was also confirmed in the present study by modulation of the activities: reduced CAT, enhanced GST and LPO occurrence even at the end of the depuration period. Some studies in fish have alerted for the high bioaccumulation potential of fipronil-sulfone compared to the parent compound (Konwick et al., 2006; Wang et al., 2016). Moreover, our results may also be suggestive of a longer persistence of fipronil-sulfone with increasing temperatures, as higher concentrations of this metabolite were present in the bile of European sea bass reared at 16 ºC than in those reared at 13 ºC However, more studies are needed to strengthen this hypothesis. If confirmed, a potential synergistic interaction between fipronil-sulfone and warmer temperatures should be taken into consideration when predicting future consequences in a global warming scenario.

Conclusions

A two-week dietary administration of the pesticide fipronil induced physiological responses in the European sea bass, as indicated by alterations in several markers. Trends on plasma and skin mucus metabolites were indicative of an increased energy

demand during fipronil exposure and after depuration. Fipronil administration also caused an oxidative-stress condition that persisted even after depuration and was accompanied by the modification of some phase I CYP-related activities and an increase of phase II GST activity. Osmoregulation and some digestive enzymes were also altered as a consequence of the pesticide administration. Chemical analyses in bile and muscle confirmed intake and clearance of fipronil (faster in muscle than in liver) but persistence of the metabolite fipronil-sulfone in bile even after the depuration period. Although a modest temperature increase of 3 ºC did not enhance fipronil effects, the persistence of fipronil-sulfone in bile at higher temperature may alert for potential synergistic effects in a CC scenario.

Conflict of interest

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

Acknowledgements

This work was financed by the Spanish Ministry of Economy, Industry and Competivity (ref CGL2016-76332-R MINECO/FEDER, UE). The Catalonian Government (Excellence Research Groups 2017SGR00902) and the Spanish Ministry of Science Innovation and Universities (RTI2018-094667-B-C21) are also acknowledged. We are indebted to L. Berdié (CCiTUB, Barcelona, Spain) for performing chemical analyses in muscle tissue and E. Martínez for helping with fish rearing. P. Dourado acknowledges a fellowship grant ref. FAPESP2017/18210-2 from Brazil. N. Montemurro acknowledges SCIEX for providing the loan instrument LC/HRMS QTOF X500R system.

References

Acena, J., Perez, S., Eichhorn, P., Solé, M., Barceló, D., 2017. Metabolite profiling of carbamazepine and ibuprofen in *Solea senegalensis* bile using high-resolution mass spectrometry. Analytical and Bioanalytical Chemistry 409, 5441–5450.

Almeida, J.R., Gravato, C., Guilhermino, L., 2015. Effects of temperature in juvenile seabass (*Dicentrarchus labrax* L.) biomarker responses and behaviour: implications for environmental monitoring. Estuaries and Coasts 38, 45–55.

Ardeshir, R.A., Zolgharnein, H., Movahedinia, A.A., Salamat, N., Zabihi, E., Regenstein, J., 2017a. Intraperitoneal fipronil effects on liver histopathological, biochemistry and morphology in Caspian kutum, *Rutilus frisii* kutum (Kamenskii, 1901). Global Journal of Environmental Science and Management-Gjesm 3, 351–362.

Ardeshir, R.A., Zolgharnein, H., Movahedinia, A., Salamat, N., Zabihi, E., 2017b. Comparison of waterborne and intraperitoneal exposure to fipronil in the Caspian white fish (*Rutilus frisii*) on acute toxicity and histopathology. Toxicology Reports 4, 348–357.

Ardeshir, R.A., Zolgharnein, H., Movahedinia, A., Salamat, N., Zabihi, E., 2018. CYP1A gene expression as a basic factor for fipronil toxicity in Caspian kutum fish. Toxicology Reports 5, 113–124.

Bencic, D.C., Villeneuve, D.L., Biales, A.D., Blake, L., Durhan, E.J., Jensen, K.M., Kahl, M.D., Makynen, E.A., Martinovic-Weigelt, D., Ankley, G.T., 2013. Effects of the insecticide fipronil on reproductive endocrinology in the fathead minnow. Environmental Toxicology and Chemistry 32, 1828–1834.

Bessey, O.A., Lowry, O.H., Brock, M.J., 1946. A method for the rapid determination of alkaline phosphatase with 5 cubic millimeters of serum. Journal of Biological Chemistry 164, 321–329.

Blanco, M., Fernandes, D., Medina, P., Blazquez, M., Porte, C., 2016. Drospirenone intake alters plasmatic steroid levels and cyp17a1 expression in gonads of juvenile sea bass. Environmental Pollution 213, 541–548.

Blazquez, M., Piferrer, F., Zanuy, S., Carrillo, M., Donaldson, E.M., 1995. Development of sex control techniques for European sea bass (*Dicentrarchus labrax* L) aquaculture: effects of dietary 17 alpha-methyltestosterone prior to sex differentiation. Aquaculture 135, 329–342.

Boltana, S., Sanhueza, N., Aguilar, A., Gallardo-Escarate, C., Arriagada, G., Valdes, J.A., Soto, D., Quinones, R.A., 2017. Influences of thermal environment on fish growth. Ecology and Evolution 7, 6814–6825.

Bonmatin, J.M., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D.P., Krupke, C., Liess, M., Long, E., Marzaro, M., Mitchell, E.A.D., Noome, D.A., Simon-Delso, N., Tapparo, A., 2015. Environmental fate and exposure; neonicotinoids and fipronil. Environmental Science and Pollution Research 22, 35–67.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Calvo, E., Simo, R., Coma, R., Ribes, M., Pascual, J., Sabates, A., Gili, J.M., Pelejero, C., 2011. Effects of climate change on Mediterranean marine ecosystems: the case of the Catalan Sea. Climate Research 50, 1–29.

Clasen, B., Loro, V.L., Cattaneo, R., Moraes, B., Lopes, T., de Avila, L.A., Zanella, R., Reimche, G.B., Baldisserotto, B., 2012. Effects of the commercial formulation

containing fipronil on the non-target organism *Cyprinus carpio*: Implications for rice - fish cultivation. Ecotoxicology and Environmental Safety 77, 45–51.

Collier, A.C., Tingle, M.D., Keelan, J.A., Paxton, J.W., Mitchell, M.D., 2000. A highly sensitive fluorescent microplate method for the determination of UDP-glucuronosyl transferase activity in tissues and placental cell lines. Drug Metabolism and Disposition 28, 1184–1186.

Crespo, M., Solé, M., 2016. The use of juvenile *Solea solea* as sentinel in the marine platform of the Ebre Delta: in vitro interaction of emerging contaminants with the liver detoxification system. Environmental Science and Pollution Research 23, 19229–19236.

Dahkqvist, A., 1970. Assay of intestinal disaccharidase. Enzymologia Biologica et Clinica 11, 52–66.

Das, P.C., Cao, Y., Cherrington, N., Hodgson, E., Rose, R.L., 2006. Fipronil induces CYP isoforms and cytotoxicity in human hepatocytes. Chemico-Biological Interactions 164, 200–214.

EFSA, 2013. Conclusion on the peer review of the pesticide risk assessment for bees for the active substance fipronil. EFSA Journal 11, 3158.

EPA, 1996. New Pesticide Fact Sheet - Fipronil; EPA 737–F–96–005; U.S. Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances, Office of Pesticide Programs, U.S. Government Printing Office Washington, DC, pp. 1–10.

Erlanger, B.F., Cohen, W., Kokowsky, N., 1961. Preparation and properties of 2 new chromogenic substrates of trypsin. Archives of Biochemistry and Biophysics 95, 271–278.

European Union. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union L 276, 20 October 2010, pp. 33–79.

European Union. Commission Implementing Regulation (EU) Nº 781/2013 of 14 August 2013 amending Implementing Regulation (EU) No 540/2011, as regards the conditions of approval of the active substance fipronil, and prohibiting the use and sale of seeds treated with plant protection products containing this active substance. Official Journal of the European Union L 219, 15 August 2013, pp. 22–25.

FAO, 2005: Cultured Aquatic Species Information Programme. *Dicentrarchus labrax*. Cultured Aquatic Species Information Programme. Text by Bagni, M. In: *FAO Fisheries and Aquaculture Department* (online). Rome. Updated 18 February 2005 (Last consulted 8 September 2019).

Fernández-Alacid, L., Sanahuja, I., Ordonez-Grande, B., Sanchez-Nuno, S., Viscor, G., Gisbert, E., Herrera, M., Ibarz, A., 2018. Skin mucus metabolites in response to physiological challenges: A valuable non-invasive method to study teleost marine species. Science of the Total Environment 644, 1323–1335.

Fernández-Alacid, L., Sanahuja, I., Ordonez-Grande, B., Sanchez-Nuno, S., Herrera, M., Ibarz, A., 2019. Skin mucus metabolites and cortisol in meagre fed acute stress-attenuating diets: Correlations between plasma and mucus. Aquaculture 499, 185–194.

Filippov, A.A., Golovanova, I.L., 2012. The effect of organic toxicants on sensitivity of intestinal glycosidases to Cu and Zn in juvenile roach. Inland Water Biology 5, 140–146.

Filippov, A.A., Golovanova, I.L., Aminov, A.I., 2013. Effects of organic pollutants on fish digestive enzymes: A review. Inland Water Biology 6, 155–160.

Ghazanfar, M., Shahid, S., Qureshi, I.Z., 2018. Vitamin C attenuates biochemical and genotoxic damage in common carp (*Cyprinus carpio*) upon joint exposure to combined toxic doses of fipronil and buprofezin insecticides. Aquatic Toxicology 196, 43–52.

Gisbert, E., Gimenez, G., Fernandez, I., Kotzamanis, Y., Estevez, A., 2009. Development of digestive enzymes in common dentex *Dentex dentex* during early ontogeny. Aquaculture 287, 381–387.

Gisbert, E., Nolasco, H., Solovyev, M., 2018. Towards the standardization of brush border purification and intestinal alkaline phosphatase quantification in fish with notes on other digestive enzymes. Aquaculture 487, 102–108.

Golovanova, I.L., Kuzmina, V.V., Chuiko, G.M., Ushakova, N.V., Filippov, A.A., 2011. Impact of polychlorinated biphenyls on the activity of intestinal proteinases and carbohydrases in juvenile roach *Rutilus rutilus* (L.). Inland Water Biology 4, 249–255.

González-Mira, A., Torreblanca, A., Hontoria, F., Navarro, J.C., Mananos, E., Varo, I., 2018. Effects of ibuprofen and carbamazepine on the ion transport system and fatty acid metabolism of temperature conditioned juveniles of *Solea senegalensis*. Ecotoxicology and Environmental Safety 148, 693–701.

Gripp, H.S., Freitas, J.S., Almeida, E.A., Bisinoti, M.C., Moreira, A.B., 2017. Biochemical effects of fipronil and its metabolites on lipid peroxidation and enzymatic antioxidant defense in tadpoles (*Eupemphix nattereri*: Leiuperidae). Ecotoxicology and Environmental Safety 136, 173–179.

Gunes, E., Yerli, S.V., 2011. Effects of Deltamethrin on Lipase Activity in Guppies (*Poecilia reticulata*). Turkish Journal of Fisheries and Aquatic Sciences 11, 473–476.

Gupta, R.C., Milatovic, Dejan, 2014. Insecticides, in: R.C. Gupta (Ed.), Biomarkers in Toxicology. Elsevier Inc., pp. 389–407.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases – First enzymatic step in mercapturic acid formation. Journal of Biological Chemistry 249, 7130–7139.

Hainzl, D., Cole, L.M., Casida, J.E., 1998. Mechanisms for selective toxicity of fipronil insecticide and its sulfone metabolite and desulfinyl photoproduct. Chemical Research in Toxicology 11, 1529–1535.

Hansen, J.W., Challinor, A., Ines, A., Wheeler, T., Moron, V., 2006. Translating climate forecasts into agricultural terms: advances and challenges. Climate Research 33, 27–41.

Holm, H., Hanssen, L.E., Krogdahl, A., Florholmen, J., 1988. High and low inhibitor soybean meals affect human duodenal proteinase activity differently: in vivo comparison with bovine serum albumin. The Journal of Nutrition, 118, 515–520.

Hooper, M.J., Ankley, G.T., Cristol, D.A., Maryoung, L.A., Noyes, P.D., Pinkerton, K.E., 2013. Interactions between chemical and climate stressors: A role for mechanistic toxicology in assessing climate change risks. Environmental Toxicology and Chemistry 32, 32–48.

Hosokawa, M., Satoh, T., 2005. Measurement of carboxylesterase (CES) activities. In: Costa, L.G., Hodgson, E., Lawrence, D.A., Ozolins, T.R., Reed, D.J., Greenlee, W.F. (Eds.), Current protocols in toxicology. John Wiley & Sons, Chapter 4, unit 4.7.

Huang, Q.T., Sheng, C.W., Jiang, J., Tang, T., Jia, Z.Q., Han, Z.J., Zhao, C.Q., 2019. Interaction of insecticides with heteromeric GABA-gated chloride channels from zebrafish *Danio rerio* (Hamilton). Journal of Hazardous Materials 366, 643–650.

Iijima, N., Tanaka, S., Ota, Y., 1998. Purification and characterization of bile salt-activated lipase from the hepatopancreas of red sea bream, *Pagrus major*. Fish Physiology and Biochemistry 18, 59–69.

IPCC, 2014*:* Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, 151 pp.

Izvekova, G.I., Solovyev, M.M., Kashinskaya, E.N., Izvekov, E.I., 2013. Variations in the activity of digestive enzymes along the intestine of the burbot *Lota lota* expressed by different methods. Fish Physiology and Biochemistry 39, 1683–1684.

Jacquin, L., Gandar, A., Aguirre-Smith, M., Perrault, A., Le Henaff, M., De Jong, L., Paris-Palacios, S., Laffaille, P., Jean, S., 2019. High temperature aggravates the effects of pesticides in goldfish. Ecotoxicology and Environmental Safety 172, 255–264.

Jiang, W.Y., Soeprono, A., Rust, M.K., Gan, J., 2014. Ant control efficacy of pyrethroids and fipronil on outdoor concrete surfaces. Pest Management Science 70, 271–277.

Konwick, B.J., Garrison, A.W., Black, M.C., Avants, J.K., Fisk, A.T., 2006. Bioaccumulation, biotransformation, and metabolite formation of fipronil and chiral legacy pesticides in rainbow trout. Environmental Science & Technology 40, 2930–2936.

Kuz'mina, V.V., Chuiko, G.M., Pavlov, D.F., 1999. Effects of DDVP, naphthalene, and cadmium on intestinal proteolytic activity in Mozambique Tilapia (*Oreochromis mossambicus* Peters). Bulletin of Environmental Contamination and Toxicology 62, 193–198.

Lalles, J.P., 2010. Intestinal alkaline phosphatase: multiple biological roles in maintenance of intestinal homeostasis and modulation by diet. Nutrition Reviews 68, 323–332.

Leghait, J., Gayrard, V., Picard-Hagen, N., Camp, M., Perdu, E., Toutain, P.L., Viguié, C., 2009. Fipronil-induced disruption of thyroid function in rats is mediated by increased total and free thyroxine clearances concomitantly to increased activity of hepatic enzymes. Toxicology 255, 38–44.

Li, H.Z., You, J., Wang, W.X., 2018. Multi-compartmental toxicokinetic modeling of fipronil in tilapia: Accumulation, biotransformation and elimination. Journal of Hazardous Materials 360, 420–427.

Lorentz, K., Gutschow, B., Renner, F., 1999. Evaluation of a direct alpha-amylase assay using 2-chloro-4-nitrophenyl-alpha-D-maltotrioside. Clinical Chemistry and Laboratory Medicine 37, 1053–1062.

Lu, D.H., Liu, D.H., Gu, X., Diao, J.L., Zhou, Z.Q., 2010. Stereoselective metabolism of fipronil in water hyacinth (*Eichhornia crassipes*). Pesticide Biochemistry and Physiology 97, 289–293.

Makrinos, D.L., Bowden, T.J., 2016. Natural environmental impacts on teleost immune function. Fish & Shellfish Immunology 53, 50–57.

Maroux, S., Louvard, D., Baratti, J., 1973. Aminopeptidase from hog intestinal brush border. Biochimica et Biophysica Acta 321, 282–295.

Maulvault, A.L., Barbosa, V., Alves, R., Custodio, A., Anacleto, P., Repolho, T., Ferreira, P.P., Rosa, R., Marques, A., Diniz, M., 2017. Ecophysiological responses of juvenile seabass (*Dicentrarchus labrax*) exposed to increased temperature and dietary methylmercury. Science of the Total Environment 586, 551–558.

Menezes, C., Leitemperger, J., Murussi, C., Viera, M.D., Adaime, M.B., Zanella, R., Loro, V.L., 2016. Effect of diphenyl diselenide diet supplementation on oxidative stress biomarkers in two species of freshwater fish exposed to the insecticide fipronil. Fish Physiology and Biochemistry 42, 1357–1368.

Mnif, W., Hassine, A.I.H., Bouaziz, A., Bartegi, A., Thomas, O., Roig, B., 2011. Effect of endocrine disruptor pesticides: a review. International Journal of Environmental Research and Public Health 8, 2265–2303.

Navarro, J.M., Paschke, K., Ortiz, A., Vargas-Chacoff, L., Pardo, L.M., Valdivia, N., 2019. The Antarctic fish *Harpagifer antarcticus* under current temperatures and salinities and future scenarios of climate change. Progress in Oceanography 174, 37–43.

Parry, R. M., Chandan, R. C., and Shahani, K. M. (1965). A rapid and sensitive assay of muramidase. Experimental Biology and Medecine 119, 384–386.

Qu, H., Ma, R.X., Wang, F., Gao, J., Wang, P., Zhou, Z.Q., Liu, D.H., 2018. The effect of biochar on the mitigation of the chiral insecticide fipronil and its metabolites burden on loach (*Misgurnus anguillicaudatus*). Journal of Hazardous Materials 360, 214–222.

Regoli, F., Giuliani, M.E., 2014. Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Marine Environmental Research 93, 106–117.

Samaras, A., Papandroulakis, N., Lika, K., Pavlidis, M., 2018. Water temperature modifies the acute stress response of European sea bass, *Dicentrarchus labrax* L. (1758). Journal of Thermal Biology 78, 84–91.

Sanahuja, I., Fernandez-Alacid, L., Sanchez-Nuno, S., Ordonez-Grande, B., Ibarz, A., 2019. Chronic cold stress alters the skin mucus interactome in a temperate fish model. Frontiers in Physiology 9.

Schiedek, D., Sundelin, B., Readman, J.W., Macdonald, R.W., 2007. Interactions between climate change and contaminants. Marine Pollution Bulletin 54, 1845–1856.

Schlenk, D., Huggett, D.B., Allgood, J., Bennett, E., Rimoldi, J., Beeler, A.B., Block, D., Holder, A.W., Hovinga, R., Bedient, P., 2001. Toxicity of fipronil and its degradation products to *Procambarus* sp.: field and laboratory studies. Archives of Environmental Contamination and Toxicology 41, 325–332.

Simon-Delso, N., Amaral-Rogers, V., Belzunces, L.P., Bonmatin, J.M., Chagnon, M., Downs, C., Furlan, L., Gibbons, D.W., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D.P., Krupke, C.H., Liess, M., Long, E., McField, M., Mineau, P., Mitchell, E.A.D., Morrissey, C.A., Noome, D.A., Pisa, L., Settele, J., Stark, J.D., Tapparo, A., Van Dyck, H., Van Praagh, J., Van der Sluijs, J.P., Whitehorn, P.R., Wiemers, M., 2015. Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. Environmental Science and Pollution Research 22, 5–34.

Simon, L.M., Laszlo, K., Kotorman, M., Vertesi, A., Bagi, K., Nemcsok, J., 1999. Effects of synthetic pyrethroids and methidation on activities of some digestive enzymes in carp (*Cyprinus carpio* L.). Journal of Environmental Science and Health Part B-Pesticides, Food Contaminants and Agricultural Wastes 34, 819–828.

Smith, E.M., Wilson, J.Y., 2010. Assessment of cytochrome P450 fluorometric substrates with rainbow trout and killifish exposed to dexamethasone, pregnenolone-16 alpha-carbonitrile, rifampicin, and beta-naphthoflavone. Aquatic Toxicology 97, 324–333.

Sokolova, I.M., Lannig, G., 2008. Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: implications of global climate change. Climate Research 37, 181–201.

Solé, M., Livingstone, D.R., 2005. Components of the cytochrome P450-dependent monooxygenase system and 'NADPH-independent benzo a pyrene hydroxylase' activity in a wide range of marine invertebrate species. Comparative Biochemistry and Physiology C-Toxicology and Pharmacology 141, 20–31.

Solé, M., Vega, S., Varo, I., 2012. Characterization of type "B" esterases and hepatic CYP450 isoenzimes in Senegalese sole for their further application in monitoring studies. Ecotoxicology and Environmental Safety 78, 72–79.

Solé, M., Fortuny, A., Mananos, E., 2014. Effects of selected xenobiotics on hepatic and plasmatic biomarkers in juveniles of *Solea senegalensis*. Environmental Research 135, 227–235.

Spain. Royal Decree 53/2013, de 1 de Febrero, por el que se establecen las normas básicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia. Boletin Oficial del Estado (BOE) nº 34, 8 de Febrero de 2013, pp. 11370–11421.

Stefani Margarido, T.C., Felicio, A.A., Rossa-Feres, D.d.C., de Almeida, E.A., 2013. Biochemical biomarkers in *Scinax fuscovarius* tadpoles exposed to a commercial formulation of the pesticide fipronil. Marine Environmental Research 91, 61–67.

Sun, L., Jin, R., Peng, Z., Zhou, Q., Qian, H., Fu, Z., 2014. Effects of trilostane and fipronil on the reproductive axis in an early life stage of the Japanese medaka (*Oryzias latipes*). Ecotoxicology 23, 1044–1054.

Tavares, M.A., Palma, I.D.F., Medeiros, H.C.D., Guelfi, M., Santana, A.T., Mingatto, F.E., 2015. Comparative effects of fipronil and its metabolites sulfone and desulfinyl on the isolated rat liver mitochondria. Environmental Toxicology and Pharmacology 40, 206–214.

Vargas-Chacoff, L., Arjona, F.J., Ruiz-Jarabo, I., Pascoa, I., Goncalves, O., del Rio, M.P.M., Mancera, J.M., 2009. Seasonal variation in osmoregulatory and metabolic parameters in earthen pond-cultured gilthead sea bream *Sparus auratus*. Aquaculture Research 40, 1279–1290.

Vassault, A., 1983. Lactate dehydrogenase, in: M.O. Bergmeyer (Ed.), Methods of enzymatic analysis, enzymes: oxidoreductases, transferases. Academic Press, New York, pp. 118–126.

Vinagre, C., Madeira, D., Narciso, L., Cabral, H.N., Diniz, M., 2012. Effect of temperature on oxidative stress in fish: Lipid peroxidation and catalase activity in the muscle of juvenile seabass, *Dicentrarchus labrax.* Ecological Indicators 23, 274–279.

Wang, X., Martinez, M.A., Wu, Q.H., Ares, I., Martinez-Larranaga, M.R., Anadon, A., Yuan, Z.H., 2016. Fipronil insecticide toxicology: oxidative stress and metabolism. Critical Reviews in Toxicology 46, 876–899.

WHO, 2009. The WHO recommended classification of pesticides by hazard and guidelines to classification: 2009. World Health Organisation (WHO).

Wu, H., Gao, C., Guo, Y., Zhang, Y., Zhang, J., Ma, E., 2014. Acute toxicity and sublethal effects of fipronil on detoxification enzymes in juvenile zebrafish (*Danio rerio*). Pesticide Biochemistry and Physiology 115, 9–14.

Xiong, D.M., Xie, C.X., Zhang, H.J., Liu, H.P., 2011. Digestive enzymes along digestive tract of a carnivorous fish *Glyptosternum maculatum* (Sisoridae, Siluriformes). Journal of Animal Physiology and Animal Nutrition 95, 56–64.

Zaugg, W.S., 1982. A simplified preparation for adenosine-triphosphatase determination in gill tissue. Canadian Journal of Fisheries and Aquatic Sciences 39, 215–217.

Zhang, B., Zhang, L., He, L.J., Yang, X.D., Shi, Y.L., Liao, S.W., Yang, S., Cheng, J.G., Ren, T.R., 2018. Interactions of Fipronil within fish and insects: experimental and molecular modeling studies. Journal of Agricultural and Food Chemistry 66, 5756–5761.

Zhao, X.L., Yeh, J.Z., Salgado, V.L., Narahashi, T., 2005. Sulfone metabolite of fipronil blocks gamma-aminobutyric acid- and glutamate-activated chloride channels in mammalian and insect neurons. Journal of Pharmacology and Experimental Therapeutics 314, 363–373.

Figure captions

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

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

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

Fig 1

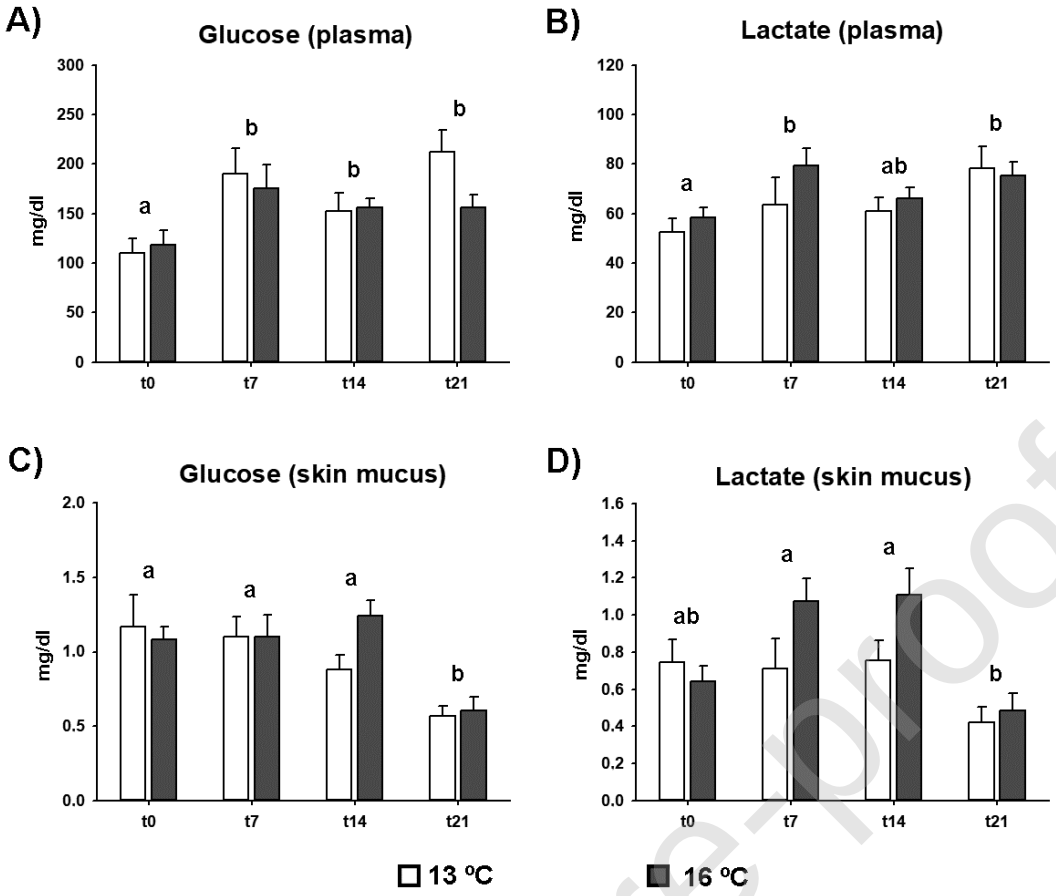


Fig 2

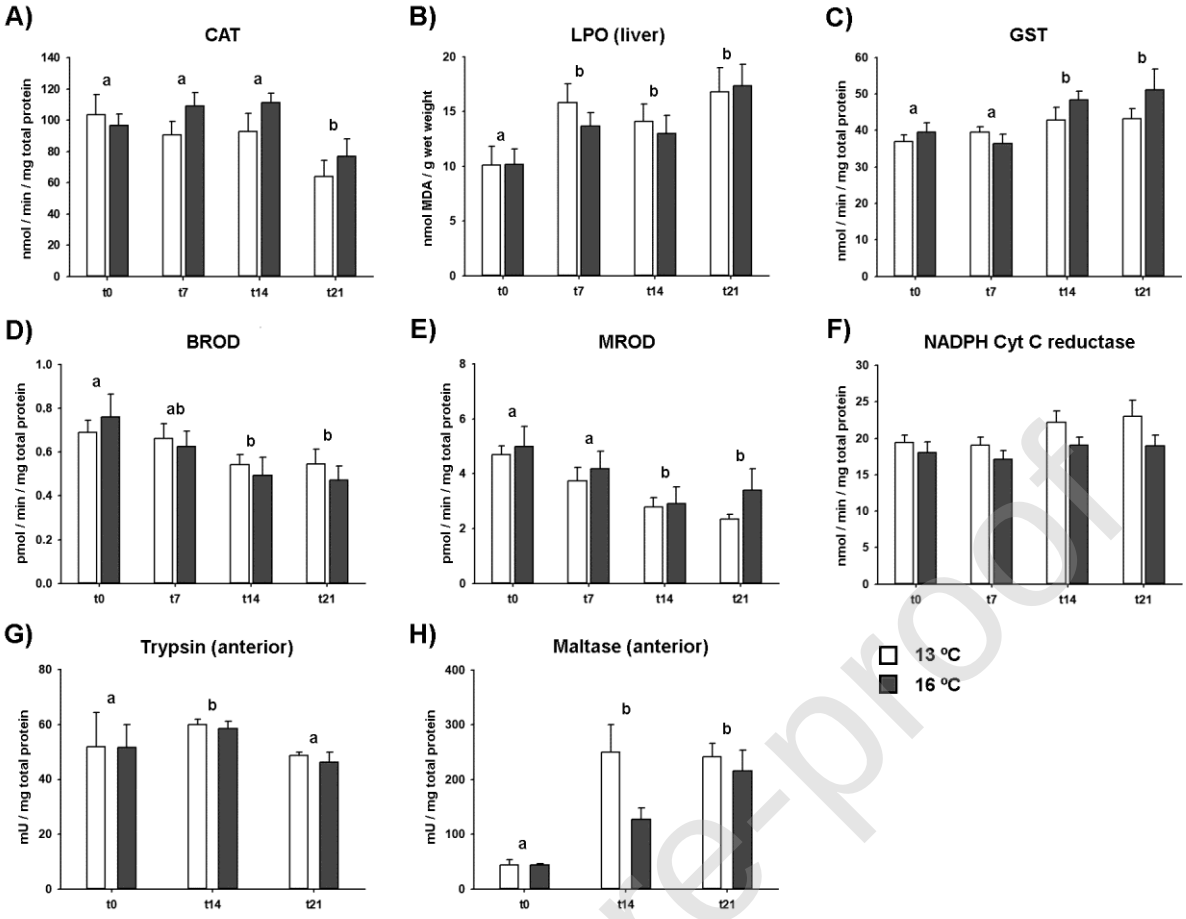
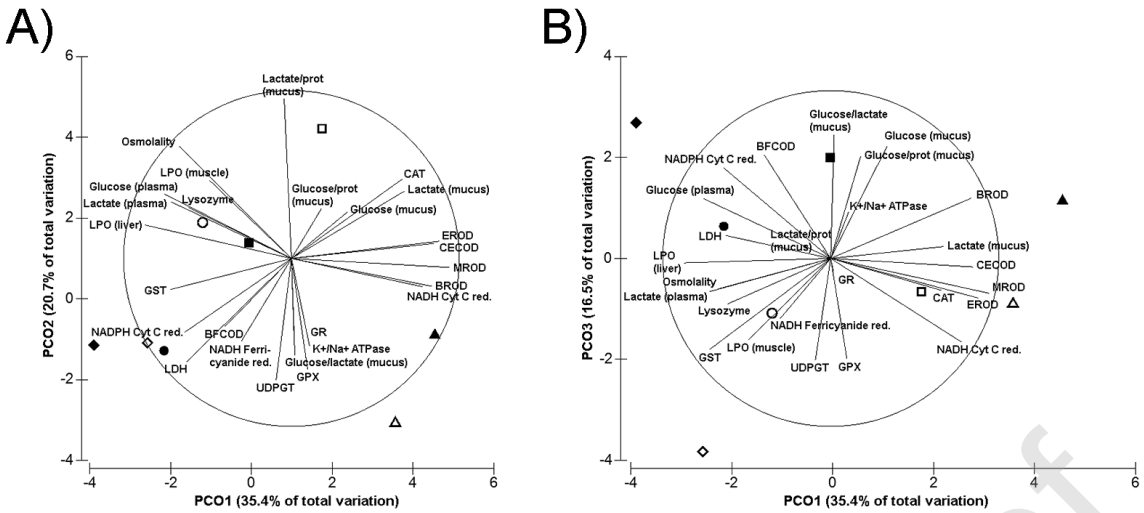


Fig 3



Tables

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

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

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

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

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