Multi-organ characterisation of B-esterases in the European sea bass

(*Dicentrarchus labrax*): effects of the insecticide fipronil at two temperatures

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Graphical abstract

Highlights

- Brain B-esterases were elevated due to fipronil exposures
- A temperature increase of 3°C only occasionally affected B-esterase activities
- Carboxylesterase measures in liver expressed higher hydrolysis with α NB
- B-esterases were present in *D. labrax* brain, muscle, mucus, plasma, liver, kidney, gills and blood
- A depuration period longer than 7-day would be needed to reach control conditions

Abstract

In fish, the study of cholinesterases (ChEs) and carboxylesterases (CEs), apart from their involvement in neural activity and xenobiotic metabolism, respectively, requires to be further explored. The European sea bass (*Dicentrarchus labrax*) was the fish model used to characterise B-esterases in several matrices and organs, as well as to assess the impacts of the insecticide fipronil at two temperatures: the natural temperature at the time of sampling (13 °C) and at 16 °C (based on climate change-related predictions for the Mediterranean region). Fipronil exerts harmful effects in non-target species; however, some countries are reluctant to implement regulations without additional evidence on their toxicity. A comprehensive study was performed in fish pre-acclimated to the two targeted temperatures for 15 days. B-esterases were evaluated in multiple samples after 7 and 14 day exposures to fipronil in feed (dose of 10 mg/kg) and after a 7-day depurative period. Based on hydrolysis rates, results showed that CEs were measurable in all matrices while ChEs were more abundant in muscle and, particularly, acetylcholinesterase (AChE) in the brain. A +3 °C increase in temperature had little influence on B-esterase activity; however, fipronil caused a significant increase in brain AChE (1.5-fold) and CE (3-fold) activities. Other matrices and organs also experienced

alterations in their B-esterase activities that could compromise their physiological functions.

Keywords: Climate change, pesticides, carboxylesterases, cholinesterases, sea bass.

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1. Introduction

Pesticides and specifically insecticides are chemicals that have been broadly used for years in agriculture to control pests and enhance crop production. Their use in agriculture has allowed improvements in food and biofuel production efficiencies to support increasing human populations and energetic demands. However, indiscriminate use of these chemicals not only had an impact on the targeted pests but also on non-target species in nearby ecosystems or more distant areas due to chemical runoff (Lushchak et al., 2018). Among the effects of problematic systemic pesticides (e.g. neonicotinoids and fipronil) in non-target species, those in birds, mammals, insects and aquatic fauna are notable, and have been reviewed as part of a monographic issue: *Worldwide Integrated Assessment of the Impact of Systemic Pesticides on Biodiversity and Ecosystems*. Due to hydrodynamic characteristics and proximity to urban sites, estuaries and fish inhabiting or temporarily nursing in this ecosystem are particularly vulnerable to anthropogenic chemicals that reach these areas through either underground filtration or direct river discharge (Cuevas et al., 2018). To improve the water quality of these highly productive ecosystems, they have been included in the framework of multiple UN Sustainable Development Goals including *Clean Water and Sanitation*, *Responsible Consumption and Production*, *Climate Action* and *Life Below Water*.

Among estuarine fish, wild and cultured specimens of the European sea bass, *Dicentrarchus labrax*, have been proposed as sentinels due to their biological and ecological features (Fernandes et al., 2008). Juveniles of this fish species have been selected to assess the effects of temperature (Vinagre et al., 2012; Almeida et al., 2015; Barbosa et al., 2017), chemicals (Fernandes et al., 2008; Blanco et al., 2016) and the combination of both (Maulvault et al., 2017) on their physiology under controlled

laboratory conditions. As characteristics of estuarine fish, juveniles of this species can tolerate large salinity and temperature gradients, and can be found upstream in some European rivers where exposure to chemicals, including pesticides is more likely to occur. The consequences of juvenile exposure to these problematic chemicals could also be inferred in other estuarine fish. *D. labrax* is the second-most farmed fish species in aquaculture in Europe (FAO, 2018), which justifies its economic interest and also its importance for human health; thus, any significant alterations in its fitness, growth or reproductive output due to any stressor could have economic or health impacts.

Biomarkers, such as activities of enzymes classed as B-esterases, namely cholinesterases (ChEs) and carboxylesterases (CEs), are well-adapted in pollution monitoring as they are highly responsive to pesticides but also to other ester-containing chemicals. However, prior to their application, there is a need to evaluate, for each sentinel species, the suitability of available commercial substrates and the most adequate tissue/organ in which to conduct the measures (Wheelock et al., 2008). ChEs are endogenous hydrolases mainly involved in neural transmission (e.g. acetylcholinesterase; AChE), while pseudocholinesterases (propionylcholinesterase; PrChE and butyrylcholinesterase; BuChE) play a less clear physiological role with a projected neurotoxic protection as well as a cell proliferation/differentiation regulation role (Wogram et al., 2001; Lockridge, 2015). An additional role for pseudocholinesterases that could gain importance under stressful conditions, or due to the presence of AChE inhibitors, would be to take over AChE tasks (Falugi and Aluigi, 2012). Moreover, the presence of AChE outside the nervous system indicates other roles for this enzyme in addition to its main implication in neural transmission (Soreq and Seidman, 2001; Pickett et al., 2017). Conversely, CEs are ubiquitously distributed in all phylogenetic groups, as well as among tissues/organs (Satoh and Hosokawa, 1998,

2006) where they may play roles yet to be unravelled. At present, it is known that CEs have a key physiological and metabolic significance as they are involved in the hydrolysis of endogenous esters, as well as of many drugs administered in ester form (Ross and Crow, 2007). Moreover, CEs have been conferred a protective role (i.e. to prevent AChE inhibition) thanks to their high stoichiometric affinity binding with organophosphorus pesticides (Wheelock et al., 2008).

In fish, although B-esterases are mainly classed as neuromuscular and metabolic enzymes, they are also present and partly characterised even in less-invasive matrices, such as plasma and the skin mucus of freshwater (Chuiko et al., 2003; Nigam et al., 2014) and marine fish (Sanahuja et al., 2019), including *D. labrax* (Brandts et al., 2018). The presence of CEs in less-invasive matrices has special interest as it allows the application of protocols that do not require the animal's sacrifice, therefore making studies more ethical as well as allowing repeated measures over time in the same individual, reinforcing traceability and reducing variability and the number of experimental fish required. Some studies have noted a promising relationship between plasma and skin mucus in the case of some metabolites and other parameters in *Argyrosomus regius* (Fernández-Alacid et al., 2019), in their responses to nanomaterials in *Sparus aurata* (Oliveira et al., 2018) and to nanoplastics in *D. labrax* (Brandts et al., 2018). Characterisation of B-esterase activity and distribution in several fish tissues/organs has been described in the marine benthic fish *Solea senegalensis* (Solé et al., 2012), the mesopelagic fish *Trachurus trachurus*, *Merluccius merluccius* and *Trisopterus luscus* (Martínez-Morcillo et al., 2019), and *S. aurata* (Soto-Mancera et al., 2020) and *D. labrax* (Varò et al., 2003), all fish species of high economic interest. Other fish species also characterised in terms of B-esterases are the reef fish *Haemulon plumieri* (Alpuche-Gual and Gold-Bouchot, 2008), the catadromous fish *Anguilla*

anguilla (Valbonesi et al., 2011) and the freshwater model *Danio rerio* (Wu et al., 2014).

Among systemic pesticides, fipronil is a broad-spectrum insecticide used worldwide that belongs to the phenylpyrazole chemical family and can be sold commercialized under many brand names (e.g. Regent800®WG, containing 80 % active ingredient). The toxicity of fipronil is based on its main mechanistic interaction with gamma-aminobutyric acid (GABA) receptors but it is also responsible for oxidative-stress and metabolic alterations (Wang et al., 2016). Fish are able to metabolise fipronil and one of the main biotransformation products, fipronil sulfone, can be even more toxic than the parental compound (Simon-Delso et al., 2015). Its use was banned by the European Union in April 2013 (Commission implementing regulation (EU) N° 781/2013) but some countries like Spain did not adhere to the directive blaming the lack of sufficient scientific evidence of its toxicity on non-target species. Up to now, fipronil toxicity evidence in fish has been provided by studies with the freshwater species *D. rerio* (Wu et al., 2014), carp, *Cyprinus carpio* (Qureshi et al., 2016) and the marine fish *D. labrax* (Dallarés et al., 2020). The latter study is complementary to the present one and demonstrated that spiked food at a concentration of 10 mg/kg fipronil (under the same commercial Regent800®WG brand) was bioaccumulated and further metabolised by European sea bass causing metabolic disturbances and gonadal histological alterations over time (Dallarés et al., 2020; Blázquez et al., 2018).

The aims of the present work were: 1) to characterise the distribution of B-esterases in different tissues of the European sea bass to reveal the most adequate tissue-biomarker descriptor, including two less-invasive matrices: plasma and skin mucus, and 2) to evaluate B-esterase responsiveness to fipronil food administration as potential

biomarkers and verify if these activities could be affected by a modest temperature increase of +3 °C, in accordance to previsions of climate change conditions for the Mediterranean region, to provide scientific evidence of toxicity in a commercial species, which could be useful to policy makers.

2. Materials and methods

2.1. Experimental design

This study is part of a larger experiment, whose water conditions, maintenance and fish characteristics are detailed in Dallarés et al. (2020). Briefly, juvenile specimens of European sea bass (*D. labrax*) of an average weight of 131.6 ± 46.1 g and reared at the IRTA (Sant Carles de la Ràpita, Spain) aquaculture facilities, were further acclimated in the experimental aquaria facilities (ZAE) of ICM-CSIC (Barcelona, Spain) for 2 weeks. They were then allowed to acclimatise for another 2 weeks to the targeted temperatures: 13 °C, the natural one at the time of the experiment in winter and 16 °C as a reasonable temperature increase predicted by IPCC (2014) to be reached by 2100 in the Mediterranean region. After these 2 weeks of acclimatisation (time 0), fipronil administration was initiated at 10 mg/kg in the food for two additional weeks and fish sampled after 7, 14 and 21 days (the last 7-day period without spiked food was considered a depuration period).

2.2. Fish Sampling

Eight fish were sampled at each temperature regime (4 fish/replicate tank) immediately before the beginning of fipronil feed administration (t0), at 7 (t7) and 14 (t14) days of feeding with spiked food and after 7 additional days of depuration (t21). Fish were fasted for 48 h before each sampling. Fish were anaesthetised with 0.2 % 2-

phenoxyethanol (Sigma-Aldrich, St. Louis, MO, USA), and sacrificed by severing their spinal cord, eviscerated, weighed and the weight of liver, gonads and visceral fat were also recorded (data shown in Dallarés et al., 2020). Skin mucus was collected as described in (Fernández-Alacid et al., 2018) using sterile glass slides from the over-lateral line in the caudal direction. Posteriorly, 1 mL of blood was withdrawn from the caudal vein using heparinised syringes. Fish organs/tissues, including liver, gonads, head kidney, brain, gills and a portion of the axial muscle were then collected. After collection, all matrices were submerged in liquid nitrogen and kept at -80°C for further analyses.

Fish were handled following 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). The procedures used were approved by the ethics committee of the Local Government of Catalonia with reference FUE-2018-00813667. All steps were aimed to minimise animal suffering.

2.3. Sample preparation for enzymatic analyses

The different samples: plasma, skin mucus, muscle, liver, head kidney, gills, gonads and brain were prepared using particular protocols and buffers as described in Dallarés et al., (2020) and summarised in supplementary table 1.

In all cases, supernatants originating from homogenisation of the above listed eight tissue/organs were aliquoted and stored at -80°C for further enzymatic determinations.

2.4. Cholinesterase activities

For ChEs measurements, three different substrates (acetylthiocholine iodide (ASCh), S-butyrylthiocholine iodide (BuSCh) and propionyl thiocholine iodide (PrSCh) at a concentration of 1 mM were used following Ellman's protocol (Ellman et al., 1961). In each microplate well, 150 μ l of 270 μ M DTNB (5,5'-dithio-bis-(2-nitrobenzoate)) were mixed with 25 μ l of sample (undiluted or appropriately diluted) and, after 2 min pre-incubation, their reaction was initiated by adding 50 μ l of the respective substrates and monitored for 5 min at 412 nm to measure AChE, BuChE and PrChE activities.

2.5. Carboxylesterase activities

Activity of CEs was measured using the substrates p-nitrophenyl acetate (pNPA) and p-nitrophenyl butyrate (pNPB) at a concentration of 1 mM each according to Hosokawa and Satoh (2005). CE measures were also made with the substrates α -naphthyl acetate (α NA), α -naphthyl butyrate (α NB) and β -naphthyl acetate (β NA) at a 250 μ M concentration each following the Mastropaolo and Yourno (1981) protocol. In each microplate well, 25 μ L of sample (undiluted or appropriately diluted) were mixed with 200 μ L of 50 mM phosphate buffer (pH = 7.4) containing the substrate at the above mentioned concentrations and the formation of nitrophenolate (for pNPA and pNPB) and naphthol (for α NA, α NB and β NA) was monitored at 405 and 235 nm, respectively for 5 min.

2.6. Protein determination

Protein content was determined by the Bradford (1976) method, adapted to microplates at 495 nm using the Bio-Rad Protein Assay reagent. An external standard

curve with bovine serum albumin (0.05 - 0.5 mg/mL) was used for total protein quantification to which express enzymatic activities.

ChEs, CEs and protein measures were done in triplicate at 25 °C at the respective wavelengths in a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Austria) and activities expressed as nmol/min/mg protein.

2.7. Statistical analyses

Normality and equality of variance were checked using Shapiro-Wilk and Levene's tests, respectively. A one-way ANOVA test was performed followed by post-hoc Bonferroni's test (if variances among groups were equal) or Dunnett's test (for unbalanced variances) to test the effect of sampling time conditions (t0, t7, t14 and t21), an indicator of fipronil exposure, on biomarker levels. In addition, general linear models (GLMs) were used to reveal the influence of temperature, fipronil exposure and the interaction between the two factors. Bivariate Pearson correlations (r_p) were used to analyse relationships between pairs of enzyme activities. Principal component analyses (PCA) were carried out to assess a global B-esterase response to fipronil exposure throughout the experiment. PCA was performed and plotted in two dimensions, and original variables were reduced into a smaller set of artificial variables, accounting for most of the variance in the original ones. All statistical analyses were performed using SPSS Statistics for Windows software, Version 22.0 (Armonk, NY: IBM Corp.).

3. Results

3.1. B-esterase activities

ChE activities (AChE, PrChE, and BuChE) were determined in liver, muscle, gills, head kidney, gonads, brain and plasma. CE activities were determined using five

commercial substrates (pNPA, pNPB, α NA, α NB and β NA) in the same matrices including skin mucus, at both temperatures (13 °C and 16 °C) and the different time conditions (t0, t7, t14 and t21). All mean activities $\pm$ Standard Error of the Mean (SEM) (n = 8) for the different conditions are provided as supplementary material in Tables S1 and S2, for ChEs and CEs, respectively. However, when comparing baseline activities among tissues for both B-esterases, data from only the natural ambient temperature (13 °C) before fipronil administration (t0) were considered (n = 8).

Out of the three ChEs (activity in nmol/min/mg protein), AChE (mean $\pm$ SEM) showed the highest activity for most tissues, particularly in brain (42.9 ± 3.1) and muscle (33.6 ± 4.1), with moderate activity in gills, gonads and liver (13.4 ± 1.6 - 9.0 ± 2.9), and the least activity in the head kidney (4.7 ± 1.1). Pseudocholinesterases showed similar hydrolysis rate trends based on tissue but varied in a narrower range than AChE. The activity mean range for PrChE was: 18.8 - 3.1 and for BChE (including plasma measures) was: 21.5 - 2.4, with muscle always displaying the highest activities (Supplementary Table S1).

The highest CE activities (in nmol/min/mg protein) were always observed in liver and plasma regardless of the substrate assayed. Selecting pNPA-CE activity, as one of the most assayed substrates, mean hydrolysis rates across matrices considering fish at t0 and 13 °C (n = 8) followed this trend: liver (119.8) > plasma (82.5) > head kidney (79.6) > brain (27.4) > gonads (24.7) > gills (12.5) > skin mucus (10.2) > muscle (8.0) (Supplementary Table S2). The same tissue trend was observed for the substrate pNPB. CE activity using α NA, as one of the most selected naphthyl substrates, also showed the highest activity in liver (159.3 ± 16.1) followed by plasma and brain, the latter being 3.5- and 5-times lower, respectively. In the remaining organs, the hydrolysis rates varied in a narrow range (25.2 - 16.4). For α NB, the pattern of mean activity

among matrices were: liver (246) > brain $\approx$ head kidney $\approx$ plasma $\approx$ muscle (34 - 20) > gills $\approx$ gonads (10 - 8). Hydrolysis rates assayed with β NA showed low activities in all matrices (range 10.1 – 5.2) except in liver, where it was 10-times higher (61.3 ± 4.8) (Supplementary Table S2).

3.2. Correlations among B-esterase activities

High Pearson correlation coefficients (presented throughout this section) were observed within the three ChEs and also among the five CE measures, and in some cases, also between the two types of B-esterases in a tissue-dependent way. Paired correlations were calculated considering all data for each tissue ($n = 64$) and detailed results are provided in Supplementary Table S3.

The liver was the organ with the strongest correlations within ChEs ($r_p > 0.9$) and among CEs ($r_p = 0.45 - 0.92$) ($p < 0.001$ in all cases) but not between the two ($p > 0.05$). Muscle and gills showed a parallel response with all correlations significant within each B-esterase group but also between them ($r_p = 0.39 - 0.98$ in muscle and $r_p = 0.31 - 0.97$ in gills). In the head kidney, ChEs were significantly correlated ($r_p > 0.87$) as well as CEs, except when using α NB as substrate. Between the two B-esterase types, correlations were mostly significant although lower when including BuChE and naphthyl-derived substrates. In gonads, correlations within B-esterase groups and between them were significant except when including BuChE and naphthyl-derived substrates in the measures, as partly seen for kidney. In plasma, due to sample limitations, only BuChE was considered for the ChE measures and showed positive significant correlations with most CE-related activities ($r_p = 0.36 - 0.49$) except when using α NB as a CE substrate ($p > 0.05$), as also noted previously for head kidney and gonads. In the brain, also due to sample limitations, less data were available for

correlations. In this tissue, AChE showed a strong and negative correlation with pseudocholinesterases ($r_p = 0.70 - 0.86$; $p > 0.05$), probably not reaching significance due to the limited number of samples considered ($n = 16$). Nonetheless, the correlation observed between BuChE and PrChE was positive and significant ($r_p = 0.968$; $p < 0.001$; $n = 16$). Correlations within CEs measured in the brain were positive and highly significant ($r_p = 0.64 - 0.95$; $p < 0.001$; $n = 64$). In the same tissue, correlations between CEs and AChE were strong ($r_p = 0.56 - 0.75$; $p < 0.001$) but not between the former and pseudocholinesterases, due to the lower number of individuals compared, as noted above. However, when using α NB as a substrate in the CE measures, the relationship to pseudocholinesterases gained significance despite the same low number of individual data ($r_p > 0.84$; $p < 0.05$; $n = 16$). In the case of skin mucus, fewer correlations were attempted also due to sample volume limitations but within the three CE substrates assayed, pNPA, pNPB and α NA, all correlations were positive and significant ($r_p > 0.62$; $p < 0.001$).

3.3. Tissue distribution of B-esterases

The relative contribution of the three ChE and five CE measures in each tissue was calculated in respect to the total B-esterase hydrolytic activity and represented as percentages in Figure 1, considering the totality of fish (2 temperatures $\times$ 4 sampling times $\times$ 8 individuals; $n = 64$). However, these individual B-esterase contributions must be considered with caution and can only be regarded as a proxy due to an imbalance in the measures considered: 3 ChEs vs. 5 CEs and the well-recognised feature of overlapping substrate specificities among commercially available substrates.

The contribution of ChE to total B-esterase activity largely varied among tissues from 4 % (liver) to 52 % (muscle). Intermediate values for ChEs contributions were

found in head kidney (6.8 %), gonads (15.7 %), brain (22.3 %) and gills (39.2 %). Even though the brain showed the highest AChE activity, it did not display the highest ChEs contribution due to the minor contribution of pseudocholinesterases. In liver, gills and muscle a balanced contribution of the three ChEs was observed while in the head kidney, gonads and, more importantly in the brain, AChE was predominant.

CE activities (which potentially accounted for five CE isoforms) were predominant in almost all tissues, except muscle. Substrate preference was evaluated by the hydrolysis rates achieved using the different substrates (as indicated in section 3.1. but emphasised in this section due to the consideration of the totality of samples; $n = 64$ vs. $n = 8$). In agreement with the highest hydrolysis rates found in the liver, CEs represented 96 % of total B-esterase activity, with partial activity using α NB (30 %) followed by pNPB (22.4 %), α NA (20.1 %), and pNPA (15.9 %), and β NA making the least contribution (7.5 %). Muscle showed an opposite trend, that is, the lowest CEs contribution, which reached 48 %. In muscle, CEs relative contribution to the total B-esterase activity based on the different substrates assayed was similar to liver although lower and narrower in percentage range (14.5 - 4.3 %) and in line with the catalytic activities (α NB > pNPB > α NA > pNPA > β NA). In gills, all CE substrates yielded similar hydrolysis rates (10 - 19 %) except β NA (5.5 %). Despite a CEs contribution in head kidney of 93.2 %, similar to liver (96 %), in the hematopoietic organ (as well as in gonads) pNPA appeared to be the most adequate substrate. It contributed 25 % to total CE activity, similar to the other substrates (about 20 % each), with β NA the substrate displaying the least contribution (7.1 %). In the brain, individual CE substrate participation was equally represented (about 18 % each), with the exception of β NA, representing 4.6 % of total CE activity. In summary, when using CE as a biomarker in European sea bass, α NB seemed to be the most adequate substrate in liver and muscle

while pNPA was more suitable in head kidney and gonad. In brain and gills, all CE substrates assayed were equally appropriate, at least in terms of measured hydrolysis rates, which in turn are indicated as tissue/organ contribution. However, responsiveness of CE measures to environmental and chemical stressors using particular substrates requires further verification.

Due to sample volume constraints limiting the number of B-esterase measures, an assessment of their partial contribution in plasma and skin mucus was not attempted and further discussion is based on published literature.

3.4. Temperature effect on B-esterase activities

The influence of a +3 °C increase in temperature on B-esterase activities was evaluated using the multiscale general linear model (GLM) analysis and results are provided as supplementary material for ChEs (Table S4) and CEs (Table S5).

A significant ($p < 0.05$) effect of temperature on the three ChE activities was observed in liver and gills, with higher activities at the higher temperature (16 °C). On the contrary, in gonads only PrChE was significantly affected by this factor, with higher activity at the lowest natural temperature (13 °C). In plasma, due to sample limitations, only BuChE was measured, revealing that the influence of rearing temperature was highly significant ($p < 0.001$), with higher activity at the lowest temperature.

CE activities were also modestly affected by rearing temperature. Occasional significant effects were observed in liver, gonad and brain (with pNPA as a substrate) and head kidney (using pNPB) with activity increases with increasing temperature except in gonadal tissue.

3.5. Fipronil effects on B-esterase activities

The consequences of fipronil exposure in the different matrices were evaluated over time using the same GLM approach and also detailed as supplementary material for ChEs (Table S4) and CEs (Table S5).

Contrary to the minor influence of temperature on the modulation of ChEs, fipronil administration over time had a highly significant effect on all ChEs in gill ($p < 0.001$), on AChE in head kidney ($p < 0.01$) and brain ($p < 0.001$) and on PrChE and BuChE in gonad ($p < 0.05$ and $p < 0.001$, respectively). Fipronil exposure over time also affected CE activities in a substrate- and tissue-dependent manner. However, apart from some occasional effects detected in different matrices using specific substrates and generally associated with moderate degrees of significance (indicated in Table S5), a consistent and relevant effect was revealed in gills and brain as a consequence of insecticide intake.

Due to the minor influence of rearing temperature and the lack of interaction between this factor and fipronil exposure on modulating CE responses, the effects of fipronil administration over time are represented integrating data from both temperatures at each exposure time ($n = 16$), both for ChEs (Fig 2.) and CEs (Fig 3). They are represented as a percentage of activity variation normalised to control values at t_0 , which correspond to baseline activity (100 %).

All three ChEs in gills were affected by fipronil administration in a similar time-dependent way: they decreased after 7- and 14-day exposures and recovered after the depuration period (t_{21}), although without full recovery to initial values (Fig 2A). In the head kidney, ChE activities decreased over time, although significant differences with respect to the control were only detected for AChE at t_{14} and t_{21} (Fig 2B). ChE activities in gonads followed a similar U pattern to gills, but in this case variations in respect to the control (100 %) were only significant for pseudocholinesterases and, after

depuration, activity was fully restored to control values (Fig 2C). Conversely, in brain, fipronil administration caused a significant increase over time in AChE activity (by 1.5-fold in respect to controls) after 14 days and remained elevated even after depuration (Fig. 2D).

As for ChEs, the strongest impact on CE activities was ascribed to fipronil exposure in a tissue-dependent way rather than varying with temperature. Effects of fipronil on liver were more significant using α NB as a substrate with increases over 25 % of control values (Fig 3A). In muscle, a decrease in activity was significant at t14 when using the substrate pNPA and at t7 using the substrates α NA and β NA, and recovery was generally observed at day 21 (Fig 3B). The U pattern over time formerly observed in gills and gonads for ChEs was repeated in gill CE (using α NA and β NA) measures after 14 days with recovery to control values after depuration (Fig 3C). As outlined for AChE in brain, this tissue experienced the greatest activity increase in CE-related measures with all assayed substrates in respect to controls (100 %) showing activities elevated three-fold in respect to the controls after 14 days and remaining high even after 7-day depuration. This increase was observed for all substrates, but was most significant using β NA (Fig. 3D).

3.5.3. Responses in less-invasive matrices

B-esterase measures were also conducted in plasma and mucus, matrices do not require fish sacrifice. However, due to the lower hydrolysis rates for most enzymatic measures and sample volume limitations, the assessment of the consequences of a temperature increase and fipronil dosage was based on a reduced selection of measures. In plasma, effects of fipronil exposure on BuChE were independently evaluated at both temperatures, as temperature was revealed by the GLM analysis as a significant factor.

No effect was seen over time due to either temperature (data not shown) or to the insecticide (Tables S1 and S4). As GLM analyses indicated, temperature did not affect CE activities; in this case data for both temperatures was gathered at each sampling time ($n = 16$). An increase in CE activity due to fipronil was observed for all substrates but only reached significance ($p < 0.05$) when using α NB as a substrate between t7 and t21 (Tables S2 and S5).

Since mucus also presented sample volume limitations, the less studied CE determinations were given priority. GLM analyses indicated no effect due to rearing temperature and thus CE activity measures were compared combining both temperatures ($n = 16$). CE measures with pNPA and pNPB substrates fluctuated similarly over time (data not shown) but they only significantly increased ($p < 0.05$) by about 50 % at day 7 (using pNPA) in respect to the control group (Tables S2 and S5). However, the variability of the measures in this viscous fluid was greater and did not vary similarly to the closely related plasma matrix. Therefore, no definitive conclusions can be drawn from the results obtained from these less-invasive measures in the present study.

3.5.6. Multivariate analysis

Figure 4 displays a two-dimensional PCA plot, using individual fish as replicate samples ($n = 64$) and including the eight B-esterase measures addressed in the study, with 50.5 % of total variance explained by the first two components. The main trend observed, related to time-exposure to fipronil, was a differentiation of pre-exposure samples (t0) along the first PCA axis with respect to samples exposed over 14 days to fipronil (t14) and those having undergone a depuration period of 7 days (t21). Samples exposed for 7 days to fipronil occupied a somewhat intermediate position.

4. Discussion

Within B-esterases, ChEs in fish have largely been studied mostly in relation to pesticide exposures, while CEs have been poorly investigated. B-esterases in fish can be partly characterised by either measuring their hydrolysis rates in several matrices and/or with the aid of particular substrates and inhibitors. To the best of our knowledge, ChEs have only been characterised in two tissues of *D. labrax* (Varò et al., 2003) although B-esterase measures have been applied in this and other fish species (Table 1). In the present study, a full B-esterases characterisation in the marine fish *D. labrax* was carried out using specific substrates in several tissues/organs including less-invasive matrices: plasma and mucus. A total of eight B-esterase measures in eight matrices were considered and related to a physical (temperature) and a chemical (fipronil) stressor in combination, given a context of climatic predictions in the Mediterranean region by the end of the present century (IPCC, 2014). The most representative B-esterases in each matrix were also targeted as a proxy of a potential key physiological role. The most sensitive combination of substrate/matrix responsive to temperature increase and fipronil exposure were identified for its potential application in toxicity assessment and field monitoring. All together this study provides laboratory evidence of fipronil toxicity in non-target species, information that could be useful to policy-makers.

4.1. B-esterase characterisation

Based on hydrolysis rates, the contribution of the different B-esterases in the 8 contrasted matrices was evaluated. In all, ChEs were more abundant in organs involved in neural transmission: muscle and brain but also in gills, as seen in other fish studies with *S. senegalensis* (Solé et al., 2012), *H. plumieri* (Alpuche-Gual and Gold-Bouchot,

2008) and *Labeo rohita* (Ghazala et al., 2014). A balanced contribution of the three ChEs was observed in muscle and gills, while AChE was dominant in brain, which is in line with the previous observations in the same species (Varò et al 2003). In other marine fish, AChE was also dominant in brain and muscle but also present in liver of *T. trachurus*, *M. merluccius* and *T. luscus* with species-dependent particularities (Martínez-Morcillo et al., 2019). Similarly, AChE was dominant in brain and muscle of *A. anguilla* in respect to liver and plasma (Valbonesi et al., 2011), as well as in *H. plumieri* (Alpuche-Gual and Gold-bouchot, 2008) but not in *L. rohita*, in which AChE and BuChE equally contributed in brain and muscle (Ghazala et al., 2014). In gonads of *D. labrax*, AChE contribution was prevalent within ChEs, although few fish studies have considered this organ in ChE measures as it has no known neurological role. However, since AChE has other attributed roles than neural transmission (see introduction section), its presence is not unexpected in gonads of *S. senegalensis* (Solé et al., 2012). ChEs are mostly considered in pesticide pollution assessment due to the high inhibition potential demonstrated *in vivo* and *in vitro* in most of the above-mentioned studies. However, recent evidences in fish propose the inclusion of CEs as biomarkers of pesticide and drug exposures (Solé et al., 2012; Ribalta et al., 2015; Martínez-Morcillo et al., 2019; Soto-Mancera et al., 2020). In terms of hydrolysis rates, CE activities in sea bass were dominant in liver closely followed by plasma and head kidney, a trend also seen in *S. aurata* (Soto-Mancera et al., 2020) and *S. senegalensis* (Solé et al., 2012). CEs were dominant in liver and plasma of *T. trachurus*, *M. merluccius* and *T. luscus* but also in muscle and brain to a lesser extent and in a species-dependent manner (Martínez-Morcillo et al., 2019).

Regarding the most adequate combination of ChE measure and matrix, derived from the present results and other fish reports, there is no question that AChE in brain is

the most suitable biomarker. However, in monitoring programs due to sampling constraints, muscle and gills could be alternatives to assess neurotoxic exposures. In the case of *D. labrax*, CE measures in the main metabolic organ (i.e. liver) and the substrates pNPB and β NA would be the most appropriate for monitoring purposes. Gonads and head kidney CE measures with pNPA should be considered if the targeted physiological endpoints are reproduction and osmoregulation, respectively. A limited number of fish studies use a battery of commercial substrates for CE measures. That is, pNPA was the only substrate used in the study with *H. plumieri* (Alpuche-Gual and Gold-Bouchot, 2008). Two substrates, pNPA and α NA, were selected in the studies with commercial marine fish species (Solé et al., 2012; Ribalta et al., 2015; Martínez-Morcillo et al., 2019). Three substrates: pNPA, pNPV (p-nitrophenyl valerate) and α NA were used in a *S. aurata* study (Soto-Mancera et al., 2020). Of all the selected CE substrates, pNPV has a log k_{ow} (2.88) comparable to pNPB (2.39), that could lead to similar associated activities. This is supported by the highest CE activities attained in *S. aurata* with pNPV, in agreement with present results in *D. labrax* using pNPB, as well as with studies with invertebrate species in which pNPB was preferred over pNPA (Solé and Sanchez-Hernandez et al., 2018; Dallarés et al., 2019). Unexpectedly, brain displayed high CE activity with all substrates except β NA, although full confirmation of adequacy should be provided by their response to the targeted stressor(s).

Regarding less-invasive matrices, BuChE and CE activities were measurable in plasma, as well as CEs in skin mucus, and could be further considered in monitoring studies. Mucus CE (with α NA and pNPA substrates) have been characterised in the freshwater fish *Cirrhinus mrigala* and showed sensitivity to pesticides (Nigam et al., 2014). CEs substrate preference observed in skin mucus of *D. labrax* agreed with substrate preferences in other fish species (Sanahuja et al., 2019). Plasmatic CEs

displayed high hydrolysis rates and were more abundant than BuChE (the main plasmatic ChE), so they could be potentially good markers in *D. labrax* (using either pNPA or pNPB as substrates) as seen in *S. aurata* (Soto-Mancera et al., 2020).

4.2. B-esterase modulation by temperature and fipronil exposure

The generally weak effect of a modest temperature increase on B-esterase responses in the present study was supported by other biomarkers in the complementary study of Dallarés et al. (2020). However, similar temperature increases (+4 °C) modified the bioaccumulation of Hg and some biochemical responses, including brain AChE in *D. labrax*, according to Maulvault et al. (2017). Behavioural, physiological and biochemical changes were also observed in the same fish species at warmer conditions by other authors, although temperature gradients were from 7 to 10 °C larger (Vinagre et al., 2012; Almeida et al., 2015). Nevertheless, and as noted Dallarés et al. (2020) a more realistic simulation of future climate change-induced environmental conditions (i.e. including alterations of salinity or pH) should be considered to truly estimate potential metabolic/physiological alterations due to this phenomenon. Despite this, the lack of an apparent synergistic effect between temperature and fipronil effects in the present study (also noted by Dallarés et al. 2020 for other biomarkers) is an interesting observation, as it is one of the multiple negative consequences that could affect living organisms given predicted climate alterations (Sokolova and Lannig, 2008).

Regarding effects of fipronil administration, brain CE measures using β NA as substrate, despite it yielded the lowest catalytic activity, was the most sensitive to fipronil. This confirms the need to consider several substrates in CE measures, as they probably associate with different enzyme isoforms. In gill, the B-esterase inhibition pattern observed might be regarded as a protective mechanism, as it is the first line of

defence to water-borne or blood circulating contaminants, and may prevent an inhibitory action in neural transmission. However, the opposed outcome of fipronil exposure observed on B-esterases in brain (activation) has a different meaning. AChE inhibition by neurotoxic chemicals is a well-known response; in this case a significant increase in all brain B-esterases (at both temperatures) could not be linked to temperature increases as formerly reported in brain AChE of *D. labrax* by Maulvault et al. (2017). In the present study, although caspases were not measured, B-esterase activity increases may be related to apoptosis as revealed in zebrafish larvae exposed to fipronil (Park, 2020). Recent evidence of the link between fipronil effects and CE activity patterns comes from a study by Jung et al. (2020) that revealed that the administration of a CE inhibitor reversed apoptosis in mouse brain. Further evidence on fipronil toxicity, comes from the biotransformation of fipronil into fipronil sulfone (considered a more toxic metabolite) over time in bile, as well as an oxidative stress condition that persistent even after depuration (Dallarés et al., 2020). Fipronil sulfone is the main fipronil metabolite in mammals and fish (Konwick et al., 2006). In rats, the parent form and the sulfone metabolite can cross the blood-brain barrier and accumulate in the brain (Cravedi et al., 2013). Moreover, exposure to fipronil triggered apoptosis in a human neural cell line (Vidau et al., 2011) and, as previously mentioned, caused overall apoptosis in early stages of development of *D. rerio* (Park, 2020).

The reported lack of α NA-CE modulation after fipronil water-borne exposure in liver, gill, brain and muscle reported in *D. rerio* could be due to the short exposure period of only 24 h (Wu et al., 2004). In contrast, a recent study with three commercial fish species including *D. labrax* exposed to microplastics revealed an oxidative stress condition in the brain associated with a two-fold AChE activity increase, while in muscle this activity was not affected (Barboza et al., 2020). The authors related the

oxidative stress condition in the brain was caused by these micro-pollutants (or any associated chemicals) to an increase in vesicle rupture and neurotransmitter leakage that required from the AChE action to fight this condition. In the present study with *D. labrax*, a similar magnitude increase in brain AChE activity was observed that was paralleled with an oxidative stress condition (Dallarés et al., 2020) and points to general brain damage (not only to GABA receptors in the case of fipronil) but to any noxious chemical able to cross the blood-brain barrier.

CEs behaviour in the two less-invasive matrices did not match, and although hydrolysis rates in mucus were up to 10 times lower than in plasma, they were clearly measurable and experienced up to 50 % increase with respect to the control after 7 days. However, this change was transient and not maintained thereafter. This fact, together with high variability in the responses observed, makes the suitability of this marker in this matrix questionable at least regarding to fipronil. Further research is needed before implement CEs measures in skin mucus as biomarkers of exposure to chemicals other than OP pesticides.

The integration of all biomarker responses into a multidimensional scale by PCA analysis allowed a clear visualisation of the joint response to fipronil simultaneously considering all matrices. This analysis clearly revealed over time changes attributable to fipronil administration, according to which important alterations occurred at t7 with respect to initial levels. These alterations increased over time and experienced a very slight recovery towards initial values at the end of the depuration period (t21), but still far from reaching pre-exposure conditions and pointing out to the need for a much longer depuration period to restore initial settings. The same deduction was reached by Dallarés et al. (2020) using different markers but in the same experimental framework.

5. Conclusions

The present results provide information on the best combination of matrix and B-esterase measure for sublethal toxicity assessment studies using the European sea bass. Despite showing the lowest hydrolysis rates, β NA proved to be the most responsive substrate for CE measures in the brain. Of all the CE substrates assayed, α NB was more appropriate for liver and muscle whereas pNPA was a good choice for plasma, gills, head kidney, gonads and skin mucus measures. The inclusion of less-invasive matrices has great potential in biomonitoring studies. However, the use of skin mucus, although non-invasive requires further validation prior to its application. Regarding to fipronil administration, the marked increase in brain B-esterase activities was associated to oxidative stress and apoptosis, with little influence of a 3 °C temperature increase. Fipronil's ability to pass the blood-brain and potentially blood-gonad barriers alert to implications for neural and hormonal signalling on the Hypothalamic–Pituitary–Interrenal axis, and therefore, on organism's homeostasis, growth, reproduction and development.

authors_statement

All authors participated in the Writing, review & editing. IS and SD also in Data curation; Formal analysis; Investigation. MS and AI in Conceptualization; Funding acquisition and Supervision.

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.

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Conflict of interest

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

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Figure headings

Figure 1. Histograms and 2-D pie charts displaying contribution of the cholinesterases (ChEs): acetylcholinesterase (AChE), propionylcholinesterase (PrChE) and butyrylcholinesterase (BuChE) and carboxylesterases (CEs) using different substrates (pNPA, pNPB, α NA, α NB and β NA) in liver, muscle, gills, head kidney, gonads and brain of the European sea bass *Dicentrarchus labrax* (n=64). Dotted portions of 2-D pie charts correspond to contribution by ChE activities, and plaid portions to contribution by CE activities. Calculations are made in respect to total hydrolysis activities using the 8 individual B-esterase measures.

Figure 2. Histograms displaying ChE activities (AChE, PrChE and BuChE) variations in percentage normalized to activity of controls (100%) in gills (A), head kidney (B), gonads (C) and brain (D) of European sea bass diet-exposed to fipronil before exposure (t0), after 7 (t7) and 14 (t14) days of exposure and after a 7-day depuration period (t21). Data from fish exposed to the two temperatures considered in the study (i.e. 13 and 16 °C) are joined in each condition. Different letters indicate significant differences among time expositions (One-way ANOVA, $p < 0.05$, $n=16$).

Figure 3. Histograms displaying CE activities using several substrates (pNPA, pNPB, α NA, α NB and β NA) variations in percentage normalized to activity of controls (100%) in liver (A), muscle (B), gills (C) and brain (D) of European sea bass diet-exposed to fipronil before exposure (t0), after 7 (t7) and 14 (t14) days of exposure and after a 7-day depuration period (t21). Data from fish exposed to the two temperatures considered in the study (i.e. 13 and 16 °C) are joined in each condition. Different letters indicate significant differences among time expositions (One-way ANOVA, $p < 0.05$, $n=16$).

Figure 4. Plot showing factors 1 and 2 of the Principal Component Analysis (PCA) applied on B-esterase activities exposed to fipronil before exposure (circles, t0) after 7 and 14 days of exposure (triangles, t7 and rhombus, t14) and after a 7-day depuration period following exposure (dashed line, t21). Individual fish data combined all B-esterase measures for a given time exposure condition.

ase activities analysed in different tissues of several fish species. Cholinesterases (ChEs): acetylcholinesterase (AChE),
 esterase (PrChE), butyrylcholinesterase (BuChE). CE substrates: p-nitrophenyl acetate (pNPA), p-nitrophenyl butyrate (pNPB),
 erate (pNPV), α -naphthyl acetate (α NA).

	Tissue	ChEs	CEs	References
<i>brax</i>	Brain, muscle	AChE	-	Almeida et al., 2015
	Brain, muscle	AChE	-	Barboza et al., 2020
	Brain	AChE	-	Maulvault et al., 2017
	Brain, muscle	AChE, PrChE, BuChE	-	Varò et al., 2003
	Liver	-	pNPA, α NA	Ribalta et al., 2015
	Skin mucus	-	pNPA, pNPB, α NA	Sanahuja et al., 2019
	Skin mucus	-	pNPA, pNPB, α NA	"
	Brain	AChE	-	Soto-Mancera et al., 2019
	Muscle, liver, heart, gills, kidney, plasma	-	pNPA, pNPV, α NA	"
<i>urus</i>	Brain, muscle	AChE	-	Barboza et al., 2019
	Brain, muscle, liver	AChE, PrChE, BuChE	pNPA, α NA	Martínez-Morcillo et al., 2019
<i>is</i>	Brain, muscle, liver, gills, kidney, plasma, gonad	AChE, PrChE, BuChE	pNPA, α NA	Solé et al., 2012
	Liver	-	pNPA, α NA	Ribalta et al., 2015
<i>ius</i>	Skin mucus	-	pNPA, pNPB, α NA	Sanahuja et al., 2019
	Brain, muscle	AChE	-	Barboza et al., 2019
<i>eri</i>	Brain, muscle, liver, gills, eye, spleen	AChE, PrChE, BuChE	pNPA	Alpuche-Gual and Goñi, 2019
<i>uccius</i>	Brain, muscle, liver	AChE, PrChE, BuChE	pNPA, α NA	Martínez-Morcillo et al., 2019
<i>as</i>	Brain, muscle, liver	AChE, PrChE, BuChE	pNPA, α NA	"
<i>abrus</i>	Liver	-	pNPA, α NA	Ribalta et al., 2015
	Liver	-	pNPA, α NA	"
<i>ostratus</i>	Liver	-	pNPA, α NA	"
	Liver	-	pNPA, α NA	"
<i>leatus</i>	Brain, muscle, liver, gills, kidney, blood	AChE, BuChE	-	Ghazala et al., 2014
	Brain, muscle, gills	AChE, BuChE	-	Wogram et al., 2001
	Liver	AChE, BuChE	pNPV	
	Brain, muscle, liver, gills	-	α NA	Wu et al., 2014
<i>a</i>	Skin mucus	-	pNPA, α NA	Nigam et al., 2014
<i>u</i>	Brain, muscle, liver, plasma	AChE, PrChE, BuChE	-	Valbonesi et al., 2011