

Aquatic Toxicology

Multi-organ characterisation of B-esterases in the European sea bass (*Dicentrarchus labrax*): effects of the insecticide fipronil at two temperatures

--Manuscript Draft--

Manuscript Number:	AQTOX-D-20-00184R2
Article Type:	Research Paper
Keywords:	climate change; pesticides; carboxylesterases; cholinesterases; sea bass
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Abstract:	<p>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 (<i>Dicentrarchus labrax</i>) 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.</p>
Suggested Reviewers:	<p>Miguel Oliveira migueloliveira@ua.pt This authors has published biomarker studies on several fish species including <i>D. labrax</i> and the same parameters we evaluate in this work. Some of his publications being included here.</p> <p>Juana Arellano juana.arellano@uca.es This researcher has conducted several studies on B-esterases and have published a similar work to ours but in sea bream. We reference her work in our manuscript.</p> <p>Salome Martinez-Morcillo martinezmorcillo@uex.es This researcher has published several papers on carboxylesterases in marine fish as well as other organisms, including several tissues characterisation.</p> <p>Bruno Nunes nunes.b@ua.pt This researcher has published many papers, including reviews on esterases in marine species.</p>

Barcelona 18/08/20

Dear Editor,

We are grateful for the opportunity given to improve the original version of the manuscript “Multi-organ characterisation of B-esterases in the European sea bass (*Dicentrarchus labrax*): effects of the insecticide fipronil at two temperatures” by Sanahuja et al.

In this second revision, the suggestions made by both reviewer’s and the Editor have been fully considered and a compromise/response has been justified when the comments may seem difficult to handle (e.g. length of the manuscript and number of references listed).

We have highlighted the main changes in yellow and 10 references from the original version have been deleted.

We hope this new version will be suitable for publication.

Thanks for your understanding.

Montse Solé

Editor and Reviewer comments:

1. In the Abstract, “the role of cholinesterases (ChEs), other than neural activity, and of carboxylesterases (CEs)” is not clear.

R: In agreement with the Referee’s comment, the sentence has been structurally changed to ensure it is clearer and now read as:

“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.”

2. In the Abstract, “European sea bass (*Dicentrarchus labrax*) was the fish model to”. Please add “used” after “fish model”

R: The term “used” has been added.

3. In the Abstract, “pre-acclimated at the two-targeted temperatures” should be “pre-acclimated to the two targeted temperatures”

R: Corrected.

4. For the responses to comment 13 of Reviewer 1, “Page 5, line 12. Re-phrase this.”, change “CEs presence” to “The presence of CEs”. Add “,” before “reinforcing traceability”.

R: Done as suggested.

5. For the responses to comment 14 of Reviewer 1, “conservative tissues” is still used in the Introduction; “non-destructive” is still used in Results and “less invasive” is used in Discussion

R: In agreement with the Referee’s comment, we normalised the mucus nomination to refer as “non-invasive”, and “less-invasive” when embracing both matrices. The terms “conservative” and “non-destructive” have been avoided.

6. p. 18 Missing full stop in “sacrifice However”.

R: The punctuation mark “.” Has been added.

7. Please add spacing before and after mathematical signs, e.g. ($rp > 0.9$) to ($rp > 0.9$), ($n = 16$) to ($n = 16$), ($p < 0.05$) to ($p < 0.05$), etc.

R: In agreement with the Referee’s comment, we add spacing before and after mathematical signs.

8. There are more than 60 citations in this MS, please double check if all the references are necessary or very important. A MS with no more than 40 citations will be highly recommended.

R: The large number of citations is the result of the MS complexity and the inclusion of several related works in Table 1, which facilitates the readers to track esterase’s works in fish. Nevertheless, we have made an effort to reduce, as much as possible, the number of bibliographic citations, without reducing the integrity and quality of the MS. Thus, the bibliography has decreased by 10 (from 66 to 56 cites), slightly lower than the average number of citations in articles published in this journal. We hope the journal will be able to make an exception given the length of the manuscript and the inclusion of a Table with published work on the topic.

9. The comments from Reviewer 2 were not successfully appended to previous decision letter. Sorry for the inconvenience caused. Please check the following comments and give responses to comments which have not been addressed in AQTOX-D-20-00184_R1.

Reviewer 2:

The manuscript by Sanahuja et al. reports on the esterase activity of various tissues of the European sea bass exposed to stress conditions, such as temperature increase and the insecticide fipronil.

The described work represents a first attempt to define the best conditions for parameter measurements and, in my opinion, is of interest to Aquat Toxicol readers.

The manuscript is well written and the text is clear enough. I suggest a minor revision: comments are listed below.

We are happy to address the comments of this second reviewer that were not included in the first revision. We appreciate his/her positive comments and the suggestions that will surely improve the final version of the manuscript. Some of his/her suggestions coincided with the 1st reviewer.

Major comments

- The mucus and the plasma are not tissues so, please, modify where required.

R: In agreement with the Referee's comment, the term "tissues" were replaced by "matrices" when mucus and plasma were included.

- The results in the para 3.1 should be better described: 5 tables rich in data are summarised in few lines. I think that a wider description can be done, stressing where significant differences are present and in which tissues/matrices.

R. In order reach a compromise with reviewer 1 comment, an effort was made to shorten the MS. We have tried to describe relevant data in the result's section 3.1 but also in the section "3.5.6. Multivariate analysis". PCA plot is a general demonstration of the different esterase's behaviour, and show how they are modified through the experiment, without being overwhelmed by the amount of data generated in this manuscript.

Page 2, line 2. "The role of cholinesterases (ChEs) in fish..."

R: This sentence, as also proposed by reviewer 1, has been modified. It was not clear to either reviewer. We hope this is improved now.

Page 2, line 12. I suggest to define the temperature clearly and replace "+3" with "16"

R: As proposed by the Referee, "+3" was replaced with "16".

Page 3, line 12. delete "has" before "not"

R: Done.

Page 3, line 17. Insert "the" after "among"

R: Done.

Page 3, line 44. "sea" without the capital initial

R: Done.

Page 3, line 51. Insert "the" after "assess"

R: Done.

Page 4, line 59. The use of "on the other hand" is not correct as the previous sentence is not introduced by "on one hand". I suggest to replace with "conversely"

R: Done as suggested.

Page 5, line 7. insert "of" after "as"

R: Done.

Page 5, line 29. "animal sacrifice"

R: As proposed, we have changes "animal sacrifice" by "animal's sacrifice".

Page 5, line 34. "the number of required experimental fish.

R: Done.

Page 6, line 7. "family and can..."

R: Done.

Page 6, line 22. "parental" instead of "parent"

R: Corrected.

Page 7, line 32 "and 16°C as a reasonable higher temperature predicted..."

R: Done.

Page 7, line 51. "before the beginning of..."

R: Included.

Page 8, line 31. Since mucus and plasma are not tissues, I suggest to modify the para title as "Sample preparation for enzymatic analyses"

R: Done as proposed.

Page 10, line 1 "homogenized"

R: As also mentioned by referee 1, we modified section 2.3 and the spelling error was corrected.

Page 10, line 9. "...of the eight samples reported above..."

R: This 2.3. section has been modified also according to former Reviewer's comment.

Page 10, line 46. "according to Hosokawa and Satoh (2005).

R: Changed.

Page 12, line 17. Replace "tissue/organs" with "samples" or "matrices", as in the discussion

R: In agreement with the Referee's comment, "matrices" was used instead of "tissue/organs" when adequate throughout the text.

Page 16, line 34. Replace "difference" with "increase"

R: Done.

Page 18, line 1. "differences with respect..."

R: The phrase has been changed.

Page 20, line 31 "tissue/organs/matrices"

R: Based on the related comments above and of referee's 1, "tissue/organs/matrices" were changed when possible in the entire MS.

Page 23, line 15. "skin", without the capital initial

R: The spelling error has been corrected.

Page 26, line 44. Insert "the" before "European"

R: Added "the" before "European".

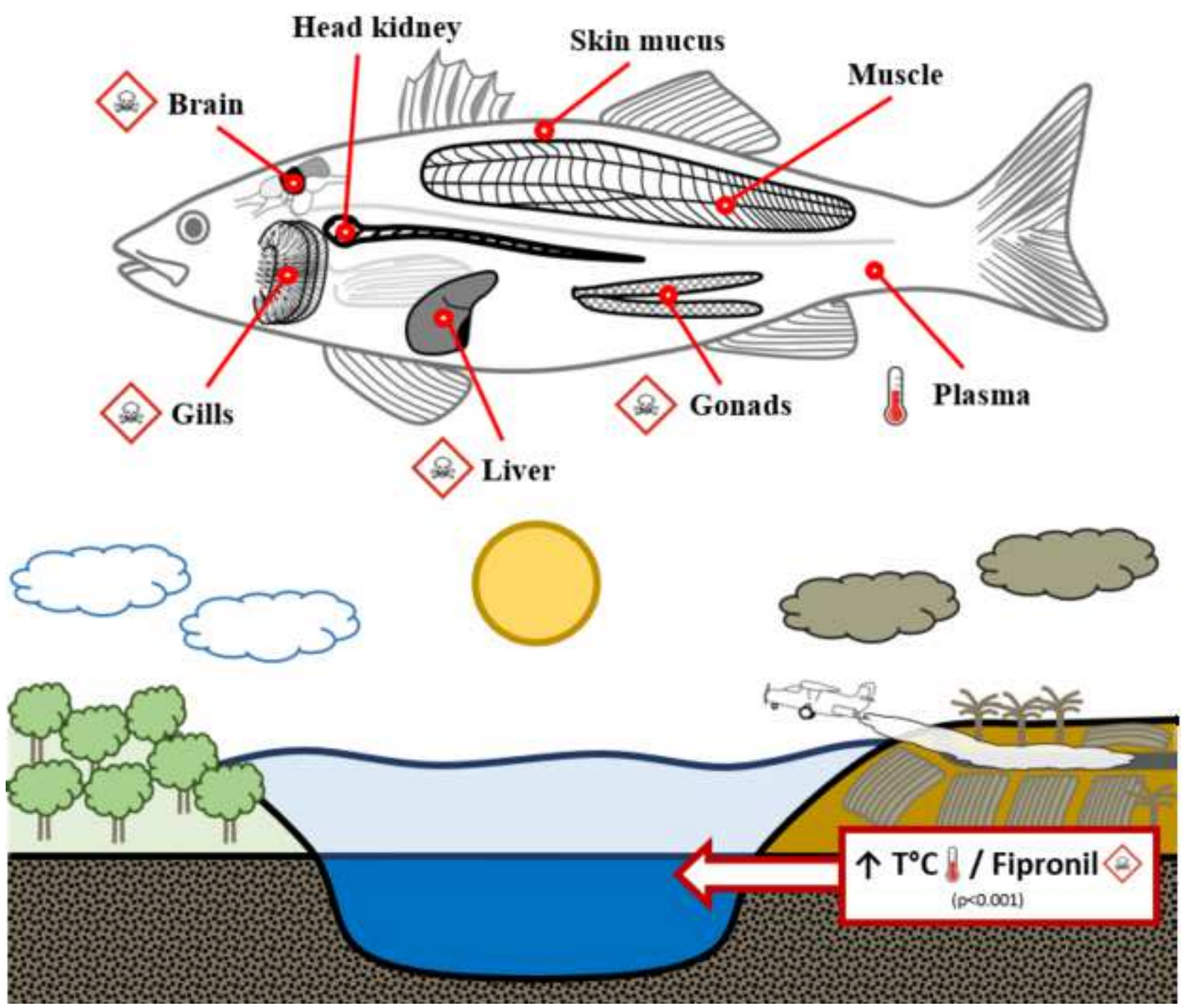
Supplementary table 4 and 5: there are some comments: please delete them.

R: We apologize for this format error that was also spotted by reviewer num 1.

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

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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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Abstract

1
2 In fish, the study of cholinesterases (ChEs) and carboxylesterases (CEs), apart from their
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4 involvement in neural activity and xenobiotic metabolism, respectively, requires to be
5
6 further explored. The European sea bass (*Dicentrarchus labrax*) was the fish model used
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8 to characterise B-esterases in several matrices and organs, as well as to assess the impacts
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10 of the insecticide fipronil at two temperatures: the natural temperature at the time of
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12 sampling (13 °C) and at 16 °C (based on climate change-related predictions for the
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14 Mediterranean region). Fipronil exerts harmful effects in non-target species; however,
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16 some countries are reluctant to implement regulations without additional evidence on
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18 their toxicity. A comprehensive study was performed in fish pre-acclimated to the two
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20 targeted temperatures for 15 days. B-esterases were evaluated in multiple samples after 7
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22 and 14 day exposures to fipronil in feed (dose of 10 mg/kg) and after a 7-day depurative
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24 period. Based on hydrolysis rates, results showed that CEs were measurable in all
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26 matrices while ChEs were more abundant in muscle and, particularly,
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28 acetylcholinesterase (AChE) in the brain. A +3 °C increase in temperature had little
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30 influence on B-esterase activity; however, fipronil caused a significant increase in brain
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32 AChE (1.5-fold) and CE (3-fold) activities. Other matrices and organs also experienced
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34 alterations in their B-esterase activities that could compromise their physiological
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36 functions.
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49 **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

1 tolerate large salinity and temperature gradients, and can be found upstream in some
2 European rivers where exposure to chemicals, including pesticides is more likely to occur.
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4 The consequences of juvenile exposure to these problematic chemicals could also be
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6 inferred in other estuarine fish. *D. labrax* is the second-most farmed fish species in
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8 aquaculture in Europe (FAO, 2018), which justifies its economic interest and also its
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10 importance for human health; thus, any significant alterations in its fitness, growth or
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12 reproductive output due to any stressor could have economic or health impacts.
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17 Biomarkers, such as activities of enzymes classed as B-esterases, namely
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19 cholinesterases (ChEs) and carboxylesterases (CEs), are well-adapted in pollution
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21 monitoring as they are highly responsive to pesticides but also to other ester-containing
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23 chemicals. However, prior to their application, there is a need to evaluate, for each
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25 sentinel species, the suitability of available commercial substrates and the most adequate
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27 tissue/organ in which to conduct the measures (Wheelock et al., 2008). ChEs are
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29 endogenous hydrolases mainly involved in neural transmission (e.g. acetylcholinesterase;
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31 AChE), while pseudocholinesterases (propionylcholinesterase; PrChE and
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33 butyrylcholinesterase; BuChE) play a less clear physiological role with a projected
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35 neurotoxic protection as well as a cell proliferation/differentiation regulation role
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37 (Wogram et al., 2001; Lockridge, 2015). An additional role for pseudocholinesterases
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39 that could gain importance under stressful conditions, or due to the presence of AChE
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41 inhibitors, would be to take over AChE tasks (Falugi and Aluigi, 2012). Moreover, the
42
43 presence of AChE outside the nervous system indicates other roles for this enzyme in
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45 addition to its main implication in neural transmission (Soreq and Seidman, 2001; Pickett
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47 et al., 2017). **Conversely**, CEs are ubiquitously distributed in all phylogenetic groups, as
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49 well as among tissues/organs (Sato and Hosokawa, 1998, 2006) where they may play
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51 roles yet to be unravelled. At present, it is known that CEs have a key physiological and
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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).

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2 Among systemic pesticides, fipronil is a broad-spectrum insecticide used
3 worldwide that belongs to the phenylpyrazole chemical family and can be sold
4 commercialized under many brand names (e.g. Regent800®WG, containing 80 % active
5 ingredient). The toxicity of fipronil is based on its main mechanistic interaction with
6 gamma-aminobutyric acid (GABA) receptors but it is also responsible for oxidative-stress
7 and metabolic alterations (Wang et al., 2016). Fish are able to metabolise fipronil and one
8 of the main biotransformation products, fipronil sulfone, can be even more toxic than the
9 parental compound (Simon-Delso et al., 2015). Its use was banned by the European Union
10 in April 2013 (Commission implementing regulation (EU) N° 781/2013) but some
11 countries like Spain did not adhere to the directive blaming the lack of sufficient scientific
12 evidence of its toxicity on non-target species. Up to now, fipronil toxicity evidence in fish
13 has been provided by studies with the freshwater species *D. rerio* (Wu et al., 2014), carp,
14 *Cyprinus carpio* (Qureshi et al., 2016) and the marine fish *D. labrax* (Dallarés et al.,
15 2020). The latter study is complementary to the present one and demonstrated that spiked
16 food at a concentration of 10 mg/kg fipronil (under the same commercial
17 Regent800®WG brand) was bioaccumulated and further metabolised by European sea
18 bass causing metabolic disturbances and gonadal histological alterations over time
19 (Dallarés et al., 2020; Blázquez et al., 2018).

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21 The aims of the present work were: 1) to characterise the distribution of B-
22 esterases in different tissues of the European sea bass to reveal the most adequate tissue-
23 biomarker descriptor, including two less-invasive matrices: plasma and skin mucus, and
24 2) to evaluate B-esterase responsiveness to fipronil food administration as potential
25 biomarkers and verify if these activities could be affected by a modest temperature
26 increase of +3 °C, in accordance to previsions of climate change conditions for the
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1 Mediterranean region, to provide scientific evidence of toxicity in a commercial species,
2 which could be useful to policy makers.
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7 **2. Materials and methods**

8 9 2.1. Experimental design

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11 This study is part of a larger experiment, whose water conditions, maintenance
12 and fish characteristics are detailed in Dallarés et al. (2020). Briefly, juvenile specimens
13 of European sea bass (*D. labrax*) of an average weight of 131.6 ± 46.1 g and reared at the
14 IRTA (Sant Carles de la Ràpita, Spain) aquaculture facilities, were further acclimated in
15 the experimental aquaria facilities (ZAE) of ICM-CSIC (Barcelona, Spain) for 2 weeks.
16 They were then allowed to acclimatise for another 2 weeks to the targeted temperatures:
17 **13 °C**, the natural one at the time of the experiment in **winter and 16 °C as** a reasonable
18 temperature increase predicted by IPCC (2014) to be reached by 2100 in the
19 Mediterranean region. After these 2 weeks of acclimatisation (time 0), fipronil
20 administration was initiated at 10 mg/kg in the food for two additional weeks and fish
21 sampled after 7, 14 and 21 days (the last 7-day period without spiked food was considered
22 a depuration period).
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43 2.2. Fish Sampling

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45 Eight fish were sampled at each temperature regime (4 fish/replicate tank)
46 immediately **before the beginning of fipronil** feed administration (t0), at 7 (t7) and 14
47 (t14) days of feeding with spiked food and after 7 additional days of depuration (t21).
48 Fish were fasted for 48 h before each sampling. Fish were anaesthetised with **0.2 %** 2-
49 phenoxyethanol (Sigma-Aldrich, St. Louis, MO, USA), and sacrificed by severing their
50 spinal cord, eviscerated, weighed and the weight of liver, gonads and visceral fat were
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1 also recorded (data shown in Dallarés et al., 2020). Skin mucus was collected as described
2 in (Fernández-Alacid et al., 2018) using sterile glass slides from the over-lateral line in
3 the caudal direction. Posteriorly, 1 mL of blood was withdrawn from the caudal vein using
4 heparinised syringes. Fish organs/tissues, including liver, gonads, head kidney, brain,
5 gills and a portion of the axial muscle were then collected. After collection, all matrices
6 were submerged in liquid nitrogen and kept at $-80\text{ }^{\circ}\text{C}$ for further analyses.
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14 Fish were handled following the Spanish regulations (RDL 53/2013), and the
15 European Directive concerning the protection of vertebrates used for experimental and
16 other scientific purposes (2010/63/EU). The procedures used were approved by the ethics
17 committee of the Local Government of Catalonia with reference FUE-2018-00813667.
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19 All steps were aimed to minimise animal suffering.
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29 2.3. Sample preparation for enzymatic analyses 30

31 The different samples: plasma, skin mucus, muscle, liver, head kidney, gills,
32 gonads and brain were prepared using particular protocols and buffers as described in
33 Dallarés et al., (2020) and summarised in supplementary table 1.
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39 In all cases, supernatants originating from homogenisation of the above listed
40 eight tissue/organs were aliquoted and stored at $-80\text{ }^{\circ}\text{C}$ for further enzymatic
41 determinations.
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49 2.4. Cholinesterase activities 50

51 For ChEs measurements, three different substrates (acetylthiocholine iodide
52 (ASCh), S-butyrylthiocholine iodide (BuSCh) and propionyl thiocholineiodide (PrSCh)
53 at a concentration of 1 mM were used following Ellman's protocol (Ellman et al., 1961).
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55 In each microplate well, 150 μl of 270 μM DTNB (5,5'-dithio-bis-(2-nitrobenzoate))
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1 were mixed with 25 μ l of sample (undiluted or appropriately diluted) and, after 2 min pre-
2 incubation, their reaction was initiated by adding 50 μ l of the respective substrates and
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4 monitored for 5 min at 412 nm to measure AChE, BuChE and PrChE activities.
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9 2.5. Carboxylesterase activities

10 Activity of CEs was measured using the substrates p-nitrophenyl acetate (pNPA)
11 and p-nitrophenyl butyrate (pNPB) at a concentration of 1 mM each according to
12
13 Hosokawa and Satoh (2005). CE measures were also made with the substrates α -naphthyl
14 acetate (α NA), α -naphthyl butyrate (α NB) and β -naphthyl acetate (β NA) at a 250 μ M
15
16 concentration each following the Mastropaolo and Yourno (1981) protocol. In each
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18 microplate well, 25 μ L of sample (undiluted or appropriately diluted) were mixed with
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20 200 μ L of 50 mM phosphate buffer (pH = 7.4) containing the substrate at the above
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22 mentioned concentrations and the formation of nitrophenolate (for pNPA and pNPB) and
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24 naphthol (for α NA, α NB and β NA) was monitored at 405 and 235 nm, respectively for 5
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39 2.6. Protein determination

40 Protein content was determined by the Bradford (1976) method, adapted to
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42 microplates at 495 nm using the Bio-Rad Protein Assay reagent. An external standard
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44 curve with bovine serum albumin (0.05 - 0.5 mg/mL) was used for total protein
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48 quantification to which express enzymatic activities.
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51 ChEs, CEs and protein measures were done in triplicate at 25 $^{\circ}$ C at the respective
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53 wavelengths in a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Austria)
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55 and activities expressed as nmol/min/mg protein.
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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 ± 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 αNA, 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 αNB, the pattern of mean activity among matrices were: liver (246) > brain ≈ head kidney ≈ plasma ≈ muscle (34 - 20) > gills ≈ gonads (10 - 8). Hydrolysis rates assayed with βNA 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

1 High Pearson correlation coefficients (presented throughout this section) were
2 observed within the three ChEs and also among the five CE measures, and in some cases,
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4 also between the two types of B-esterases in a tissue-dependent way. Paired correlations
5 were calculated considering all data for each tissue ($n = 64$) and detailed results are
6 provided in Supplementary Table S3.
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11 The liver was the organ with the strongest correlations within ChEs ($r_p > 0.9$) and
12 among CEs ($r_p = 0.45 - 0.92$) ($p < 0.001$ in all cases) but not between the two ($p > 0.05$).
13 Muscle and gills showed a parallel response with all correlations significant within each
14 B-esterase group but also between them ($r_p = 0.39 - 0.98$ in muscle and $r_p = 0.31 - 0.97$ in
15 gills). In the head kidney, ChEs were significantly correlated ($r_p > 0.87$) as well as CEs,
16 except when using α NB as substrate. Between the two B-esterase types, correlations were
17 mostly significant although lower when including BuChE and naphthyl-derived
18 substrates. In gonads, correlations within B-esterase groups and between them were
19 significant except when including BuChE and naphthyl-derived substrates in the
20 measures, as partly seen for kidney. In plasma, due to sample limitations, only BuChE
21 was considered for the ChE measures and showed positive significant correlations with
22 most CE-related activities ($r_p = 0.36 - 0.49$) except when using α NB as a CE substrate (p
23 > 0.05), as also noted previously for head kidney and gonads. In the brain, also due to
24 sample limitations, less data were available for correlations. In this tissue, AChE showed
25 a strong and negative correlation with pseudocholinesterases ($r_p = 0.70 - 0.86$; $p > 0.05$),
26 probably not reaching significance due to the limited number of samples considered ($n =$
27 16). Nonetheless, the correlation observed between BuChE and PrChE was positive and
28 significant ($r_p = 0.968$; $p < 0.001$; $n = 16$). Correlations within CEs measured in the brain
29 were positive and highly significant ($r_p = 0.64 - 0.95$; $p < 0.001$; $n = 64$). In the same
30 tissue, correlations between CEs and AChE were strong ($r_p = 0.56 - 0.75$; $p < 0.001$) but
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1 not between the former and pseudocholinesterases, due to the lower number of individuals
2 compared, as noted above. However, when using α NB as a substrate in the CE measures,
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4 the relationship to pseudocholinesterases gained significance despite the same low
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6 number of individual data ($r_p > 0.84$; $p < 0.05$; $n = 16$). In the case of skin mucus, fewer
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8 correlations were attempted also due to sample volume limitations but within the three
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10 CE substrates assayed, pNPA, pNPB and α NA, all correlations were positive and
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12 significant ($r_p > 0.62$; $p < 0.001$).
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19 3.3. Tissue distribution of B-esterases 20

21 The relative contribution of the three ChE and five CE measures in each tissue
22 was calculated in respect to the total B-esterase hydrolytic activity and represented as
23 percentages in Figure 1, considering the totality of fish (2 temperatures \times 4 sampling
24 times \times 8 individuals; $n = 64$). However, these individual B-esterase contributions must
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26 be considered with caution and can only be regarded as a proxy due to an imbalance in
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28 the measures considered: 3 ChEs vs. 5 CEs and the well-recognised feature of overlapping
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30 substrate specificities among commercially available substrates.
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39 The contribution of ChE to total B-esterase activity largely varied among tissues
40 from 4 % (liver) to 52 % (muscle). Intermediate values for ChEs contributions were found
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42 in head kidney (6.8 %), gonads (15.7 %), brain (22.3 %) and gills (39.2 %). Even though
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44 the brain showed the highest AChE activity, it did not display the highest ChEs
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46 contribution due to the minor contribution of pseudocholinesterases. In liver, gills and
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48 muscle a balanced contribution of the three ChEs was observed while in the head kidney,
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50 gonads and, more importantly in the brain, AChE was predominant.
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56 CE activities (which potentially accounted for five CE isoforms) were
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58 predominant in almost all tissues, except muscle. Substrate preference was evaluated by
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1 the hydrolysis rates achieved using the different substrates (as indicated in section 3.1.
2 but emphasised in this section due to the consideration of the totality of samples; n = 64
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4 vs. n = 8). In agreement with the highest hydrolysis rates found in the liver, CEs
5 represented 96 % of total B-esterase activity, with partial activity using α NB (30 %)
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7 followed by pNPB (22.4 %), α NA (20.1 %), and pNPA (15.9 %), and β NA making the
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9 least contribution (7.5 %). Muscle showed an opposite trend, that is, the lowest CEs
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11 contribution, which reached 48 %. In muscle, CEs relative contribution to the total B-
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13 esterase activity based on the different substrates assayed was similar to liver although
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15 lower and narrower in percentage range (14.5 - 4.3 %) and in line with the catalytic
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17 activities (α NB > pNPB > α NA > pNPA > β NA). In gills, all CE substrates yielded similar
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19 hydrolysis rates (10 - 19 %) except β NA (5.5 %). Despite a CEs contribution in head
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21 kidney of 93.2 %, similar to liver (96 %), in the hematopoietic organ (as well as in gonads)
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23 pNPA appeared to be the most adequate substrate. It contributed 25 % to total CE activity,
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25 similar to the other substrates (about 20 % each), with β NA the substrate displaying the
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27 least contribution (7.1 %). In the brain, individual CE substrate participation was equally
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29 represented (about 18 % each), with the exception of β NA, representing 4.6 % of total
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31 CE activity. In summary, when using CE as a biomarker in European sea bass, α NB
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33 seemed to be the most adequate substrate in liver and muscle while pNPA was more
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35 suitable in head kidney and gonad. In brain and gills, all CE substrates assayed were
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37 equally appropriate, at least in terms of measured hydrolysis rates, which in turn are
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39 indicated as tissue/organ contribution. However, responsiveness of CE measures to
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41 environmental and chemical stressors using particular substrates requires further
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43 verification.
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1 Due to sample volume constraints limiting the number of B-esterase measures, an
2 assessment of their partial contribution in plasma and skin mucus was not attempted and
3 further discussion is based on published literature.
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9 3.4. Temperature effect on B-esterase activities

10 The influence of a +3 °C increase in temperature on B-esterase activities was
11 evaluated using the multiscale general linear model (GLM) analysis and results are
12 provided as supplementary material for ChEs (Table S4) and CEs (Table S5).
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18 A significant ($p < 0.05$) effect of temperature on the three ChE activities was
19 observed in liver and gills, with higher activities at the higher temperature (16 °C). On the
20 contrary, in gonads only PrChE was significantly affected by this factor, with higher
21 activity at the lowest natural temperature (13 °C). In plasma, due to sample limitations,
22 only BuChE was measured, revealing that the influence of rearing temperature was highly
23 significant ($p < 0.001$), with higher activity at the lowest temperature.
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33 CE activities were also modestly affected by rearing temperature. Occasional
34 significant effects were observed in liver, gonad and brain (with pNPA as a substrate) and
35 head kidney (using pNPB) with activity increases with increasing temperature except in
36 gonadal tissue.
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46 3.5. Fipronil effects on B-esterase activities

47 The consequences of fipronil exposure in the different matrices were evaluated
48 over time using the same GLM approach and also detailed as supplementary material for
49 ChEs (Table S4) and CEs (Table S5).
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55 Contrary to the minor influence of temperature on the modulation of ChEs,
56 fipronil administration over time had a highly significant effect on all ChEs in gill ($p <$
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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

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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).

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2 Since mucus also presented sample volume limitations, the less studied CE
3 determinations were given priority. GLM analyses indicated no effect due to rearing
4 temperature and thus CE activity measures were compared combining both temperatures
5 (n = 16). CE measures with pNPA and pNPB substrates fluctuated similarly over time
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7 (data not shown) but they only significantly increased (p < 0.05) by about 50 % at day 7
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9 (using pNPA) in respect to the control group (Tables S2 and S5). However, the variability
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11 of the measures in this viscous fluid was greater and did not vary similarly to the closely
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13 related plasma matrix. Therefore, no definitive conclusions can be drawn from the results
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15 obtained from these less-invasive measures in the present study.
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24 3.5.6. Multivariate analysis

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26 Figure 4 displays a two-dimensional PCA plot, using individual fish as replicate
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28 samples (n = 64) and including the eight B-esterase measures addressed in the study, with
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30 50.5 % of total variance explained by the first two components. The main trend observed,
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32 related to time-exposure to fipronil, was a differentiation of pre-exposure samples (t0)
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34 along the first PCA axis with respect to samples exposed over 14 days to fipronil (t14)
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36 and those having undergone a depuration period of 7 days (t21). Samples exposed for 7
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38 days to fipronil occupied a somewhat intermediate position.
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45 4. Discussion

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47 Within B-esterases, ChEs in fish have largely been studied mostly in relation to
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49 pesticide exposures, while CEs have been poorly investigated. B-esterases in fish can be
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51 partly characterised by either measuring their hydrolysis rates in several matrices and/or
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53 with the aid of particular substrates and inhibitors. To the best of our knowledge, ChEs
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55 have only been characterised in two tissues of *D. labrax* (Varò et al., 2003) although B-
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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

1 Gold-bouchot, 2008) but not in *L. rohita*, in which AChE and BuChE equally contributed
2 in brain and muscle (Ghazala et al., 2014). In gonads of *D. labrax*, AChE contribution
3 was prevalent within ChEs, although few fish studies have considered this organ in ChE
4 measures as it has no known neurological role. However, since AChE has other attributed
5 roles than neural transmission (see introduction section), its presence is not unexpected
6 in gonads of *S. senegalensis* (Solé et al., 2012). ChEs are mostly considered in pesticide
7 pollution assessment due to the high inhibition potential demonstrated *in vivo* and *in vitro*
8 in most of the above-mentioned studies. However, recent evidences in fish propose the
9 inclusion of CEs as biomarkers of pesticide and drug exposures (Solé et al., 2012; Ribalta
10 et al., 2015; Martínez-Morcillo et al., 2019; Soto-Mancera et al., 2020). In terms of
11 hydrolysis rates, CE activities in sea bass were dominant in liver closely followed by
12 plasma and head kidney, a trend also seen in *S. aurata* (Soto-Mancera et al., 2020) and *S.*
13 *senegalensis* (Solé et al., 2012). CEs were dominant in liver and plasma of *T. trachurus*,
14 *M. merluccius* and *T. luscus* but also in muscle and brain to a lesser extent and in a species-
15 dependent manner (Martínez-Morcillo et al., 2019).

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36 Regarding the most adequate combination of ChE measure and matrix, derived
37 from the present results and other fish reports, there is no question that AChE in brain is
38 the most suitable biomarker. However, in monitoring programs due to sampling
39 constrains, muscle and gills could be alternatives to assess neurotoxic exposures. In the
40 case of *D. labrax*, CE measures in the main metabolic organ (i.e. liver) and the substrates
41 pNPB and β NA would be the most appropriate for monitoring purposes. Gonads and head
42 kidney CE measures with pNPA should be considered if the targeted physiological
43 endpoints are reproduction and osmoregulation, respectively. A limited number of fish
44 studies use a battery of commercial substrates for CE measures. That is, pNPA was the
45 only substrate used in the study with *H. plumieri* (Alpuche-Gual and Gold-Bouchot,
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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

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

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30 Regarding effects of fipronil administration, brain CE measures using β NA as
31 substrate, despite it yielded the lowest catalytic activity, was the most sensitive to fipronil.
32 This confirms the need to consider several substrates in CE measures, as they probably
33 associate with different enzyme isoforms. In gill, the B-esterase inhibition pattern
34 observed might be regarded as a protective mechanism, as it is the first line of defence to
35 water-borne or blood circulating contaminants, and may prevent an inhibitory action in
36 neural transmission. However, the opposed outcome of fipronil exposure observed on B-
37 esterases in brain (activation) has a different meaning. AChE inhibition by neurotoxic
38 chemicals is a well-known response; in this case a significant increase in all brain B-
39 esterases (at both temperatures) could not be linked to temperature increases as formerly
40 reported in brain AChE of *D. labrax* by Maulvault et al. (2017). In the present study,
41 although caspases were not measured, B-esterase activity increases may be related to
42 apoptosis as revealed in zebrafish larvae exposed to fipronil (Park, 2020). Recent
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1 evidence of the link between fipronil effects and CE activity patterns comes from a study
2 by Jung et al. (2020) that revealed that the administration of a CE inhibitor reversed
3 apoptosis in mouse brain. Further evidence on fipronil toxicity, comes from the
4 biotransformation of fipronil into fipronil sulfone (considered a more toxic metabolite)
5 over time in bile, as well as an oxidative stress condition that persistent even after
6 depuration (Dallarés et al., 2020). Fipronil sulfone is the main fipronil metabolite in
7 mammals and fish (Konwick et al., 2006). In rats, the parent form and the sulfone
8 metabolite can cross the blood-brain barrier and accumulate in the brain (Cravedi et al.,
9 2013). Moreover, exposure to fipronil triggered apoptosis in a human neural cell line
10 (Vidau et al., 2011) and, as previously mentioned, caused overall apoptosis in early stages
11 of development of *D. rerio* (Park, 2020).
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26 The reported lack of α NA-CE modulation after fipronil water-borne exposure in
27 liver, gill, brain and muscle reported in *D. rerio* could be due to the short exposure period
28 of only 24 h (Wu et al., 2004). In contrast, a recent study with three commercial fish
29 species including *D. labrax* exposed to microplastics revealed an oxidative stress
30 condition in the brain associated with a two-fold AChE activity increase, while in muscle
31 this activity was not affected (Barboza et al., 2020). The authors related the oxidative
32 stress condition in the brain was caused by these micro-pollutants (or any associated
33 chemicals) to an increase in vesicle rupture and neurotransmitter leakage that required
34 from the AChE action to fight this condition. In the present study with *D. labrax*, a similar
35 magnitude increase in brain AChE activity was observed that was paralleled with an
36 oxidative stress condition (Dallarés et al., 2020) and points to general brain damage (not
37 only to GABA receptors in the case of fipronil) but to any noxious chemical able to cross
38 the blood-brain barrier.
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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 CE 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

1 great potential in biomonitoring studies. However, the use of skin mucus, although non-
2 invasive requires further validation prior to its application. Regarding to fipronil
3 administration, the marked increase in brain B-esterase activities was associated to
4 oxidative stress and apoptosis, with little influence of a 3 °C temperature increase.
5 Fipronil's ability to pass the blood-brain and potentially blood-gonad barriers alert to
6 implications for neural and hormonal signalling on the Hypothalamic–Pituitary–
7 Interrenal axis, and therefore, on organism's homeostasis, growth, reproduction and
8 development.
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10 **Acknowledgements**

11 This work was financed by the Spanish Ministry of Economy, Industry and Competivity
12 (ref CGL2016-76332-R MINECO/FEDER, UE). Thank you to M. Blázquez and P.
13 Dourado for their valuable contribution in the experimental set-up and E. Gisbert from
14 IRTA (Sant Carles de la Ràpita, Spain) for providing the fish.
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17 **Conflict of interest**

18 The authors of the present study declare that they have no conflict of interest.
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1 **Figure headings**

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

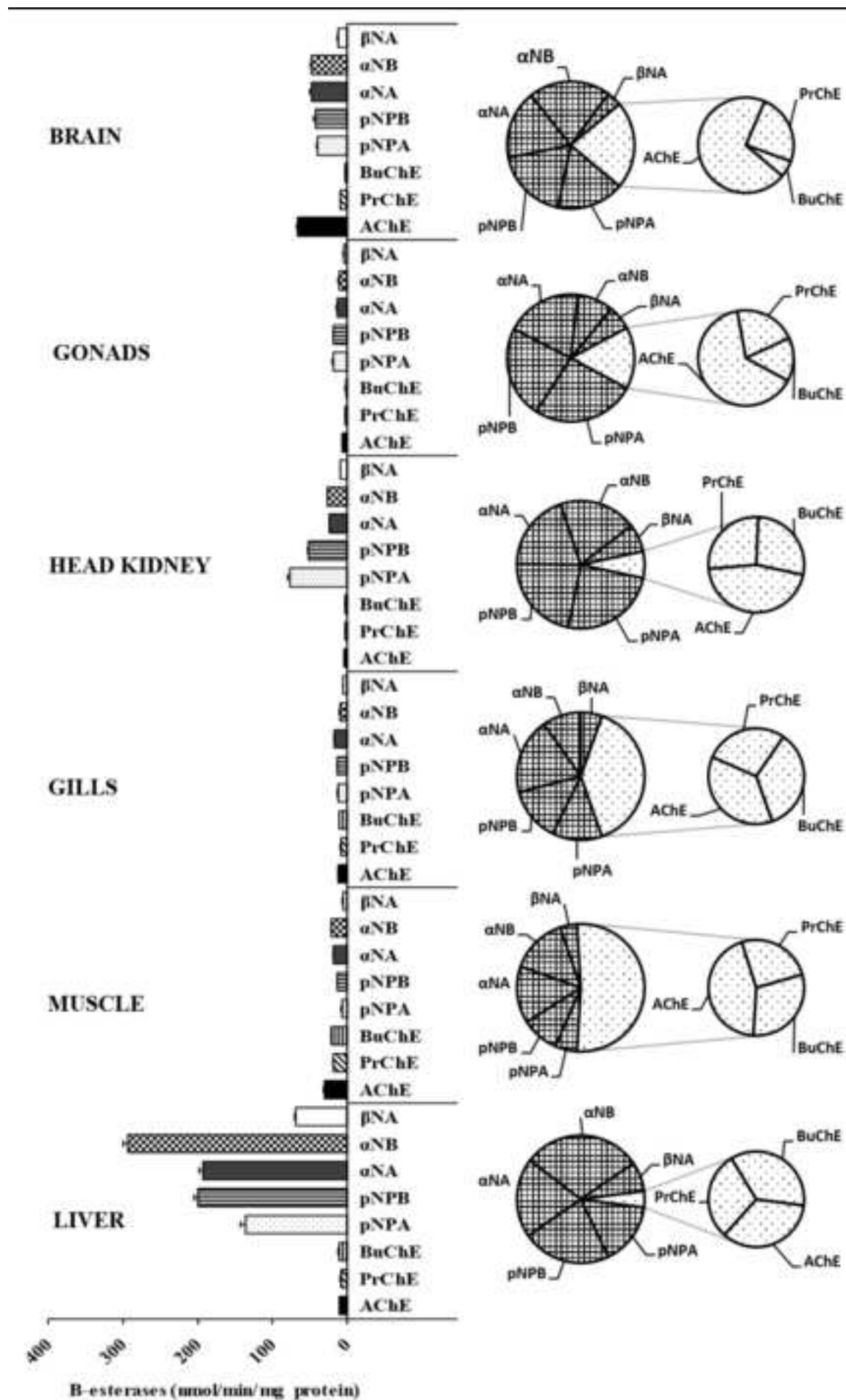
10 **Figure 2.** Histograms displaying ChE activities (AChE, PrChE and BuChE) variations in
11 percentage normalized to activity of controls (100%) in gills (A), head kidney (B), gonads
12 (C) and brain (D) of European sea bass diet-exposed to fipronil before exposure (t0), after
13 7 (t7) and 14 (t14)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

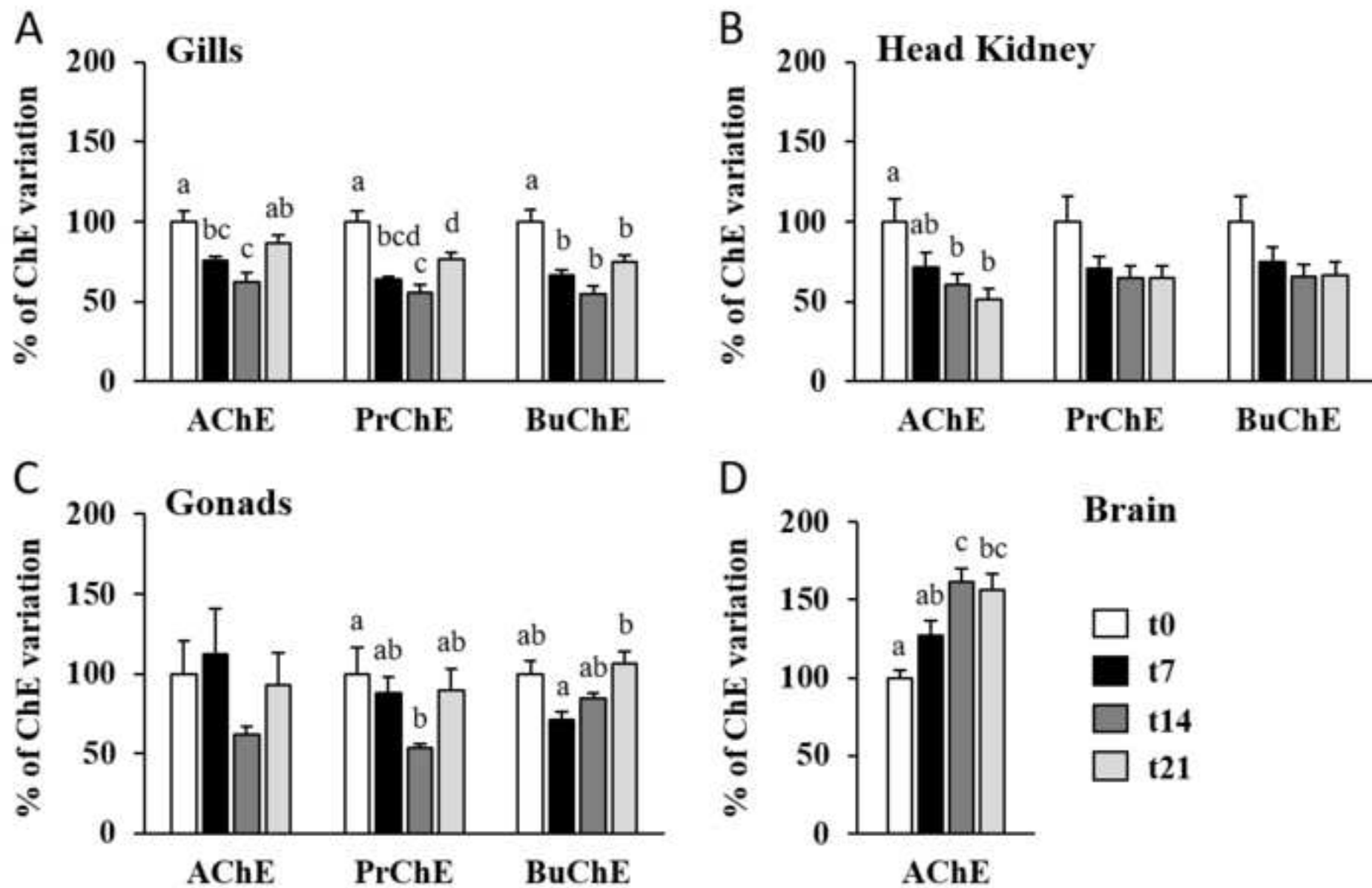
17 **Figure 3.** Histograms displaying CEs activities using several substrates (pNPA, pNPB,
18 α NA, α NB and β NA) variations in percentage normalized to activity of controls (100%)
19 in liver (A), muscle (B), gills (C) and brain (D) of European sea bass diet-exposed to
20 fipronil before exposure (t0), after 7 (t7) and 14 (t14) days of exposure and after a 7-day
21 depuration period (t21). Data from fish exposed to the two temperatures considered in the

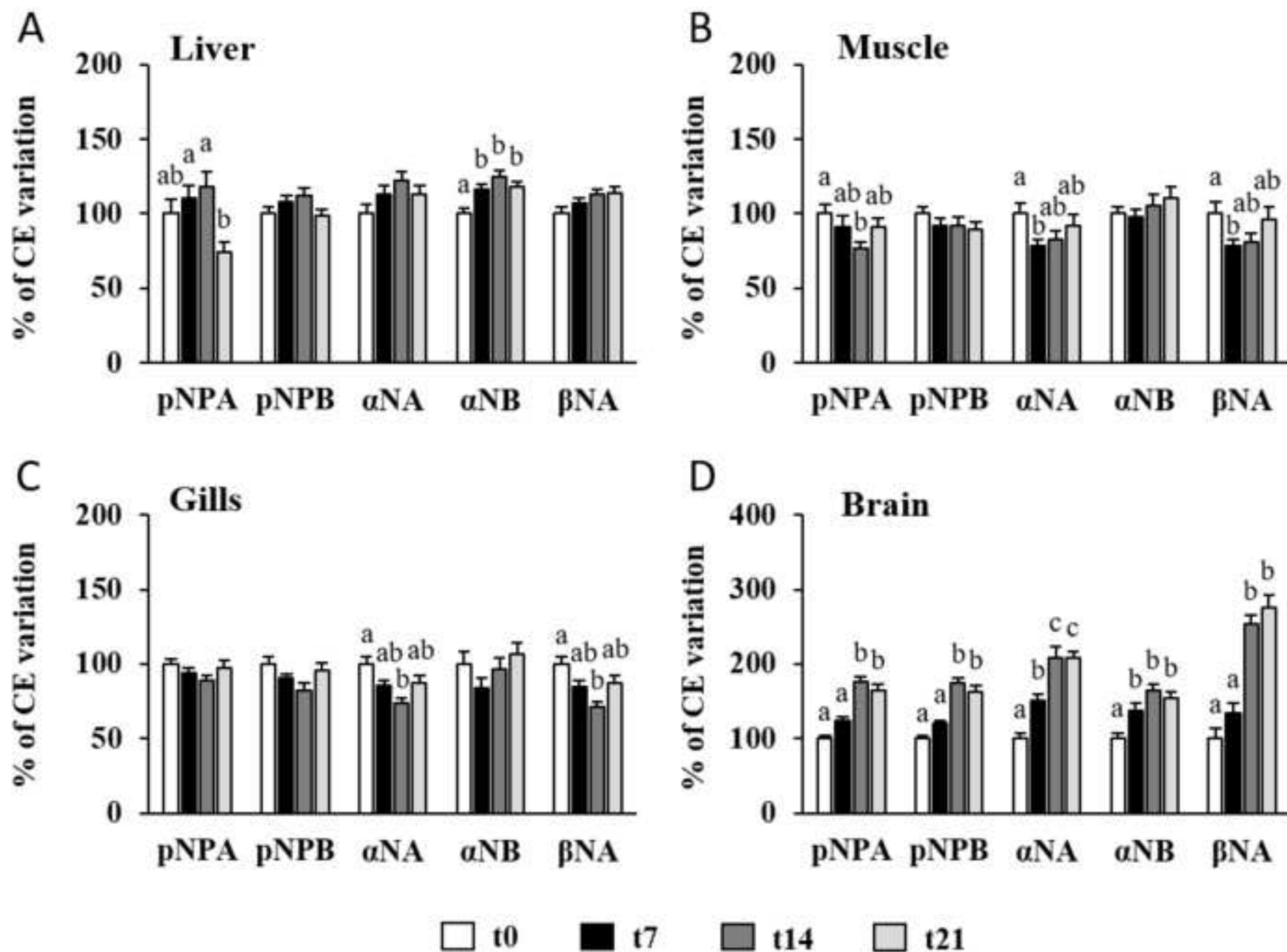
22 study (i.e. 13 and 16 °C) are joined in each condition. Different letters indicate significant
23 differences among time expositions (One-way ANOVA, $p < 0.05$, $n=16$).

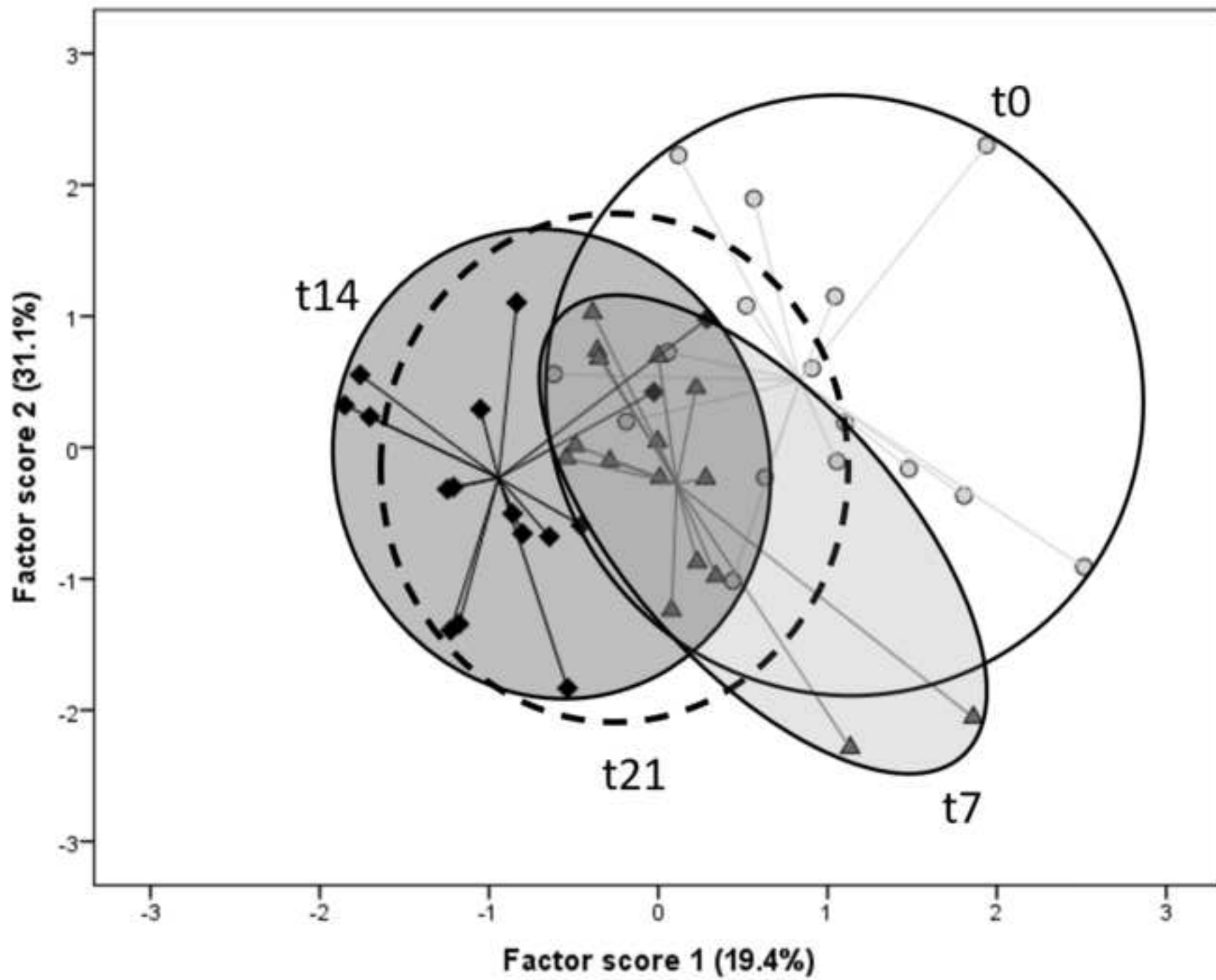
24 **Figure 4.** Plot showing factors 1 and 2 of the Principal Component Analysis (PCA)
25 applied on B-esterase activities exposed to fipronil before exposure (circles, t0) after 7
26 and 14 days of exposure (triangles, t7 and rhombus, t14) and after a 7-day depuration
27 period following exposure (dashed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

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Declaration of interests


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

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

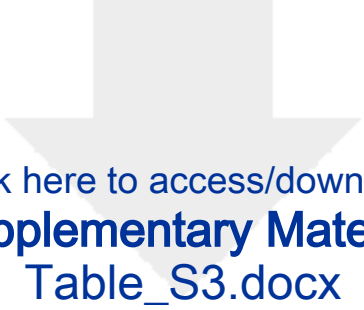
All authors participated in the Writing, review & editing. IS and SD also in Data curation; Formal analysis; Investigation. MS and AI in Conceptualization; Funding acquisition and Supervision.




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