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Interactive comment on "Ocean acidification dampens warming and contamination effects on the physiological stress response of a commercially important fish" *by* Eduardo Sampaio et al.

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Referee #2 (Manoela Orte, PhD)

General comments

"The interactive effects of acidification, warming and the presence of the metal Hg was assessed in the Fish Argyrosomus regius. Bioaccumulation of Hg was measured in different organs of the fish and sublethal toxic responses were also analyzed by the use of biomarkers. The topic is highly relevant since research regarding global change issues

should preferably focus on a multi-stressors approach. Furthermore, mercury is an important persistent contaminant found in coastal environments around the world and information regarding its interactive toxicological effects with other parameters such as acidification and warming are of great value. In general, the writing is clear and the data obtained is interesting. However, some issues regarding the methodological approach used are not well explained and there are some information at the results and discussion section that should be included. Therefore I recommend that the authors perform the suggested corrections before the article is published."

Response: We thank the referee for her comments and suggested terminology which helped to contextualize our manuscript better and improve the overall scientific outcomes found. We have addressed the lack of methodological procedures and hope that we have reached the publication standards upheld by the referee. We have accepted most of the referee's suggestions and, below, discuss each comment in a point-by-point manner. Please note that Page and Line numbers now correspond to the marked up version of the manuscript.

Specific comments

Comment #1: "The focus of the study is the evaluation of toxic responses of the metal Hg in a global change scenario. It is mentioned that concentration of Hg was chosen according to environmental measurements, however data on the range of toxic concentrations of this metal to this species or other fish species is not included. Considering that the article uses an ecotoxicological approach and therefore it is based on dose-response concentration it is crucial that more details on this subject is included, such as values of toxicity for fishes and environmental values within contaminated and non contaminated areas, especially in the area where the study was conducted."

Response: Mercury concentrations chosen for this study were based on levels of contamination found in contaminated coastal areas (specifically the extensively studied contaminated estuary of Aveiro, Portugal) for species that are natural prey ofthe mea-

gre (e.g. Cardoso et al., 2014; Nunes et al., 2008). Not exclusive to the Eastern Atlantic coast, these mercury concentrations can also be found in other areas globally (e.g. Kannan et al., 1998). We thank the reviewer for pointing out the need for contextualization and have added: "Given our dietary option, ecologically relevant MeHg concentrations were chosen based on levels (low contamination, $\sim$0.12 mg kg-1 wet weight (ww); and high contamination, $\sim$1.6 mg kg-1ww found in common A. regius prey species from contaminated coastal areas (Cardoso et al., 2014; Kannan et al., 1998; Nunes et al., 2008). The pellets given to fish allocated to non-contaminated and contaminated treatments had approximately 0.60 $\pm$ 0.01 mg kgâĄżÂź dry weight (dw) and 8.02 $\pm$ 0.01 mg kgâĄżÂź dw of MeHg, respectively, which were considered to mimick the concentrations found in the field (see Maulvault et al., 2016, 2017). Feed composition, manufacturing and MeHg spiking processes were executed as described by Maulvault et al. (2016)." (Page 4/5, Lines 30-32/1-6)

References Cardoso, P. G., Pereira, E., Duarte, A. C. and Azeiteiro, U. M.: Temporal characterization of mercury accumulation at different trophic levels and implications for metal biomagnification along a coastal food web, Mar. Pollut. Bull., 87(1), 39–47, doi:10.1016/j.marpolbul.2014.08.013, 2014. Kannan, K., Smith Jr., R. G., Lee, R. F., Windom, H. L., Heitmuller, P. T., Macauley, J. M. and Summers, J. K.: Distribution of Total Mercury and Methyl Mercury in Water , Sediment , and Fish from South Florida Estuaries, Arch. Environ. Contam. Toxicol., 34, 109–118, doi:10.1007/s002449900294, 1998. Nunes, M., Coelho, J. P., Cardoso, P. G., Pereira, M. E., Duarte, A. C. and Pardal, M. A.: The macrobenthic community along a mercury contamination in a temperate estuarine system (Ria de Aveiro, Portugal), Sci. Total Environ., 405(1–3), 186–194, doi:10.1016/j.scitotenv.2008.07.009, 2008.

Comment #2: "In the discussion section, comparative results of mercury accumulation and biomarker response are missing. The study of Biomarkers is quite complex as responses can be influenced by many parameters. In this sense, there are several studies on biomarker response to mercury in the literature. Such studies should also

be mentioned to provide information on the sensitivity of this species comparing to others, as well as to know the relevance of the used Hg concentration."

Response: The authors would like to point out that we have already synthetized some of the literature available on how these stressors prompt oxidative stress response system in the Introduction when we present the reasoning underpinning our approach (Page 2/3, Lines 26-30/1-15). We would also like to highlight that the reason we did not use a comparative Hg toxicity approach was that it is not the main aim of our work. Using the same MeHg contaminated feed, our group has recently published (inclusively this year) other experimental works where we compare the accumulation and toxicological effects (namely on oxidative stress and other enzymes) of mercury with what is described in the general literature (mainly Maulvault et al., 2017; but see also Maulvault et al., 2016 and Sampaio et al., 2016). Our main goal was to disentangle how the triple interaction of warming, acidification and mercury can modulate organism physiology (mainly through oxidative stress), and help predict fish physiological status in future ocean conditions. It was not our intention to give emphasis on mercury effects per se. Furthermore, from our perspective, the most important finding in the manuscript is that acidification counteracted the effects of both mercury contamination and warming. Thus, if we had to set a hierarchy of stressor "importance" to be explained, acidification would be on the first place, not mercury contamination. Moreover, what is important and novel in the present work is not the isolated stressors, but the interactions between them. However, taking the referee's comment into account, we do agree that it would be useful to better contextualize our study. Thus, following this suggestion, we compared these results with other studies where interactions between Hg and climate stressors were assessed: "Moreover, to cope with oxidative stress, A. regius displayed enhanced CAT, SOD and GST activities under contaminated and warming scenarios, which is in line with previous studies reporting an enhanced anti oxidative stress response in fish (Maulvault et al., 2017; Pimentel et al., 2015; Vieira et al., 2009)." (Page 11, Lines 25-29) "Increased CO2 (co-occuring with Hg contamination) is linked to upregulation of the lysosome-autophagy pathway, which is responsible for removing damaged pro-

teins and organelles, effectively reducing oxidative stress (Wang et al., 2017). This mechanism may contribute to alleviate not only Hg induced stress, but also warming-related oxidative stress." (Pages11/12, Lines 31/1-3)

References Maulvault, A. L., Custodio, A., Anacleto, P., Repolho, T., Pousao, P., Nunes, M. L., Diniz, M., Rosa, R. and Marques, A.: Bioaccumulation and elimination of mercury in juvenile seabass (Dicentrarchus labrax) in a warmer environment, Environ. Res., 149, 77–85, doi:10.1016/j.envres.2016.04.035, 2016. Maulvault, A. L., Barbosa, V., Alves, R., Custódio, A., Anacleto, P., Repolho, T., Pousão Ferreira, P., Rosa, R., Marques, A. and Diniz, M.: Ecophysiological responses of juvenile seabass (Dicentrarchus labrax) exposed to increased temperature and dietary methylmercury, Sci. Total Environ., 586, 551–558, doi:10.1016/j.scitotenv.2017.02.016, 2017. Sampaio, E., Maulvault, A. L., Lopes, V. M., Paula, J. R., Barbosa, V., Alves, R., Pousão-Ferreira, P., Repolho, T., Marques, A. and Rosa, R.: Habitat selection disruption and lateralization impairment of cryptic flatfish in a warm, acid, and contaminated ocean, Mar. Biol., 163(10), 217, doi:10.1007/s00227-016-2994-8, 2016.

Comment #3: "In the abstract, (page 1 line 20), introduction (page 3 line 20) and methodology (page 4 line 23) pCO2 concentration is given as 1100 μatm, while the actual value used was 1500 μatm. Please correct."

Response: The authors would like to clarify that 1100 μatm was the difference between both CO2 levels used (400 and 1500 μatm), i.e. delta (Δ) CO2 = 1100 μatm. The presentation rationale follows that used for presenting temperature effects: we used 19 and 23 °C, i.e. delta (Δ) T = 4 °C.

Comment #4: "The fishes were taken from an aquaculture station. Were the physico-chemical parameters measured at the station? This is relevant to known the levels of pH and temperature that organisms were acclimated at the long-term."

Response: Physico-chemical parameters at the aquaculture station were maintained under normal levels of ambient pH ($\sim$8.00) and seawater temperatures registered at

that time of the year (19 °C), which we used to serve as our control parameters. We have added this information in the text: "Juvenile Argyrosomus regius (n âL'Č 100; Fig. 5) (mean $\pm$ SD; total weight: 4.26 $\pm$ 2.8 g; total length: 6.30 $\pm$ 1.2 cm) from EPPO - IPMA (Estação Piloto de Piscicultura de Olhão – Instituto Português do Mar e da Atmosfera, Portugal) where fish were maintained under standard summer season environmental parameters (pH = 8.0 and 19 °C). In August 2014, fish were transported to the facilities of Laboratório Marítimo da Guia (LMG, MARE, Faculdade de Ciências, Universidade de Lisboa)." (Page 3/4, Lines 28-30/1-2)

Comment #5:"Page 4 Line 5- Ammonia levels is an important issue at toxicity tests, especially with fishes, as it can interfere on the toxic responses. Authors mention that ammonia (along with nitrate and nitrite) levels were kept within recommended levels. How was this performed? What are the recommended levels? Please give more details."

Response: We apologize for not having provided more detail on these matters. Specifically: Ammonia (NH3/NH4+), nitrite (NO2-) and nitrate (NO3-) concentrations were daily checked (Colorimetric kits, Aquamerk, Germany), and kept below detectable levels (i.e. NH3/NH4+ < 0.25 mg l-1; NO2- < 0.10 mg l-1; NO3- < 0.2 mg l-1). They were kept such low levels by a continuous seawater flux, and by the biological filter described (Page 4, Lines 2-5). As detailed in the Methods section (Page 4, Lines 5-9), each experimental unit (or recirculatory aquatic system, RAS) was a semi-closed system with a constant seawater flux (complete turnover rate in 24h) precisely to maintain environmental parameters such as salinity and nutrients. We have added the pertinent information in the text: "To prevent fluctuations in environmental parameters, each RAS worked as a semi-closed system, with constant low flow external water input (flux > 2 l h-1; 50 l tank turnover rate = 24 h). Consequently, ammonia (NH3/NH4+), nitrite (NO2-) and nitrate (NO3-) concentrations were daily checked (Colourimetric kits, Aquamerk, Germany), and kept below detectable levels (i.e. NH3/NH4+ < 0.25 mg l-1; NO2- < 0.10 mg l-1; NO3- < 0.20 mg l-1), and salinity was kept at 35.0 $\pm$ 1.0 (V2 Refractometer,

TMC Iberia, Portugal)." (Page 4, Lines 5-12).

Comment #6:"Salinity should be given as psu or without unit."

Response: We have removed units from salinity measurements.

Comment #7:"Page 4 line 13- Please give more details on alkalinity measurements, such as the equipment used, storage of samples, the use of certified materials. . ."

Response: We have added the requested information for alkalinity and pHT: "Seawater carbonate system speciation (Table S1) was calculated once every week from pHtotal scale (pHT) and total alkalinity. pHT was quantified via a Metrohm pH meter (826 pH mobile, Metrohm, Filderstadt, Germany) connected to a glass electrode (Schott IoLine, SI analytics, $\pm$ 0.001) and calibrated against TRIS–HCl (TRIS) and 2-aminopyridine-HCl (AMP; Mare, Liège, Belgium) seawater buffers (Dickson et al., 2007). Total alkalinity was measured spectrophotometrically (wavelength = 595 nm; UV-1800 Shimadzu, Japan) through base neutralization by formic acid and a pH sensitive dye (bromophenol blue), following Sarazin et al. (1999). Total dissolved inorganic carbon (CT), pCOâĆĆ and aragonite saturation were calculated using CO2SYS software (Lewis and Wallace, 1998), with dissociation constants from Mehrbach et al. (1973) as refitted by Dickson and Millero (1987)." (Page 4, Lines 20-28).

Comment #8:"Page 4 Line 20- The method for mercury contamination is confusing. MeHg exposure was performed by food intake and fished were fed two to three times a day. How was the difference between food intakes measured? Authors states that ingestion decreased due to changes in metabolism, but how was this measured? Where is this result? How much mercury was given as total in the experiment? How much of this metal remain dissolved in the water column?"

Response: We address each question below: Differences in food intake were not measured, as rare uneaten pellets were removed together with faeces (Page 5, Lines 8-9) and were not weighted. Thus, we have removed changes in food intake as the main un-

derlying mechanism for differences Hg accumulation and, following further comments from Referee 1, have changed our rationale to a more broader perspective: "Instead, our results support recent studies demonstrating that hypercapnia dampens Hg accumulation in marine organisms (Li et al., 2017; Sampaio et al., 2016; Wang et al., 2017). There are several possible reasons which may underpin such an interaction, encompassing digestive (reduced digestive efficiency, reduced uptake through the gut membrane, reduced appetite, increased Hg depuration) and molecular (competition between Hg and H+ ions for binding sites, impacts on Hg plasma transport, lower phospholipidic membrane permeability) mechanisms (Li et al., 2017). A recent study has also found that the lysosome-autophagy pathway was up-regulated by combined exposure to Hg and increased CO2, enabling better animal fitness which may potentially reduce Hg accumulation and toxicity (Wang et al., 2017). In addition, taking into account that the occurrence of both warming and acidification changes physiological thresholds (Christensen et al., 2011; Harley et al., 2006; Rosa et al., 2013; Rosa and Seibel, 2008), a degree of metabolic depression may also play a role on decreasing HgT accumulation (Dijkstra et al., 2013; Sampaio et al., 2016)." (Page 11, Lines 4-16) Fish were fed 2-3 times a day, but the amount of food per day was fixed at 1% mean fish weight: 4.26 g (as specified in Page 5, line 8/9) * 0.01 = 42.6 mg. Since there were 30 experimental days, then: 42.6 mg * 30 d =1278 mg or 0.001278 kg feed per fish In the pellet we have approximately 8.28 mg of HgT per Kg of food (dry weight), thus: 8.28 * 0.001278 = 0.0106 mg of HgT were given per fish, at the end of the 30-day trial. We have added the following information in the text: "..at the end of 30 days, each fish was given approximately 0.0106 mg of HgT." (Page 5, Line 10/11) Previous studies using the same food pellet manufacturing and MeHg spiking process have found that no mercury was leeched into the water column with this feed (below detectable levels; Maulvault et al., 2016).

References Maulvault, A. L., Custodio, A., Anacleto, P., Repolho, T., Pousao, P., Nunes, M. L., Diniz, M., Rosa, R. and Marques, A.: Bioaccumulation and elimination of mercury in juvenile seabass (Dicentrarchus labrax) in a warmer environment, Environ. Res.,

149, 77–85, doi:10.1016/j.envres.2016.04.035, 2016.

Comment #9: “In the experimental set-up, the setup “IV” is the same as the setup “II”, 19 âŮęC, 400 pCO2 μatm and contaminated feed (MeHg: 8.02 mg kgÂ′z; HgT: 8.28 mg kgÂ′z). Setup IV should be 19 âŮęC, 1500μatm and contaminated feed.”

Response: We have corrected the characteristics of setup iv): “19 °C, 1500 pCO2 μatm and contaminated feed”

Comment #10: “In the methodology section, it is mentioned that Reference material was also used to validate measurements of metal content. However, results of recovery percentage in not given. Please include this data as it validates the measurements.”

Response: We have included a new table (Table S1), where we include this information:

Standard reference material Total Hg Present work DORM-4* 0.390 $\pm$ 0.025 Certified value 0.410 $\pm$ 0.055

Comment #11: “Page 8 line 20-25 concentration of Hg was lower in muscle but concentration in liver and gills was actually the same considering error between replicates.”

Response: Indeed our p-value comparing levels in Liver & Gill was 0.181 and we have corrected the sentence, removing the implicated difference between HgT accumulation in the liver and the gills: “Hg concentration was lower in the muscle compared to the other two organs analyzed (Muscle & Liver / Muscle & Gills, p < 0.001, GLM Analysis in Table 1, Figure 1a).” (Page 9, Lines 18-20)

Comment #12: “Figure 1d the 400 and 1500 μatm are inverted”

Response: Corrected.

Comment #13: “Page 8 line27- As expected, catalase activity was affected by mercury contamination, but was this biomarker affected by pCO2 also? What about warming? This is briefly mentioned in the discussion section, but the results are not given.”

Response: As we have detailed in the Methods section: “Best model selection fit for our data was found using the Akaike Information Criterion (AIC), a widespread indicator that balances model complexity with model quality of fitness (Quinn and Keough, 2002). Thus, models were simplified and factors that did not influence data variation were removed.” (Page 8, Lines 5-8) In other words, using the AIC we can remove factors and interactions that do not help in explaining the data, but only add noise to the analysis. Thus, we can safely say that warming did not have an effect on CAT activity since the AIC excluded this factor from the analysis completely. As for increased CO2, the AIC did include it in the model, which means that it has influence over our data, but that influence is not significant (as we usually set an α = 0.05 in biological statistics and our analysis yielded a p = 0.116 for CO2 * MeHg). It is important to state that there is a continuous argument between statisticians over what is relevant to include or not in the discussion of this type of analysis. In our opinion, given the consistent effects on the rest of the antioxidant and physiological defense response machinery, we felt it was important to mention that an effect of CO2 in shaping CAT activity is a possibility, maybe just not detected on this study. However, we acknowledge that it was a non-significant effect. “While it is worth mentioning that increased CO2 played a minor role in CAT activity (non-significant, p = 0.116), regarding the other enzymes, hypercapnia as a sole stressor significantly augmented antioxidant activity.” (Page 11, Lines 28-30)

Comment #14: “While the values for Hsp70 are given in each organ analyzed, the results for the other biomarkers are not specified. Were they measured only in the liver or other parts? Please include this information in the results and also in the methodology.”

Response: Unfortunately we did not have enough tissue to perform enzymatic assays for oxidative stress in the liver and gills. Mercury concentration determination required almost the totally of these organs, which left us only enough sample for heat shock protein response (requires only a small tissue). Thus, the rest of the enzymatic assays were all performed in the muscle. As requested, we have added this information throughout the text, including figure captions: “As an end-product of oxidative stress,

malondialdehyde (MDA) concentration was used as a proxy to assess extent of lipid peroxidation in the muscle." (Page 6, Lines 25-26) "Catalase activity in the muscle was assessed through an adaptation of the method described by Johansson and Borg (1988)." (Page 7, Line 8) "SOD activity in the muscle was determined following the nitro blue tetrazolium (NBT) method adapted from Sun et al. (1988)."(Page 6, Line 19-20) "GSTactivity in the muscle was determined according to the procedure described by Habig et al. (1974) and optimized for 96-well microplate (Sigma Technical Bulletin, GST Assay Kit CS0410)." (Page 7, Lines 2-4) "Heat shock protein (Hsp70/Hsc70) content in the muscle, liver and gills was assessed by Enzyme-Linked Immunoabsorbent Assay (ELISA) protocol adapted from Njemini et al. (2005)." (Page 8, Lines 13-14) "Subsequently, lipid peroxidation and oxidative stress were measured in the muscle tissue. A significant antagonistic effect. . ." (Page 9, Lines 23) "Figure 2. Malondialdehyde (MDA) build-up concentrations (mean $\pm$ SE) in A. regius muscle driven by an interaction" (Page 2, Line 5) "Figure 3. a) Catalase (CAT) enzyme activities (mean $\pm$ SE) driven by MeHg contamination (Non-contaminated and Contaminated). b) Superoxide dismutase (SOD) activities (mean $\pm$ SE) in A. regius muscle. . ." (Page 24, Line 5-6) "Figure 4. Glutathione S-Transferase (GST) activities (mean $\pm$ SE) in A. regius muscle driven by:" (Page 25, Line 3)

Comment #15: "Page 9 lines 15-20 the information "However, our AIC-chosen best model indicated that mercury may diminish organism Fulton condition" is contradictory to what is mentioned on the results: "Fulton condition (K) did not show any significant differences between treatments (MeHg, $p > 0.05$, GLM analysis in Table 1)."""

Response: We have removed this statement. "The present study showed that Hg contamination, ocean warming and acidification interactively affected fish physiology at sublethal levels, i.e. zero mortality and also no effects on Fulton condition were registered." (Page 10, Lines 14-19)

Technical corrections:

Technical correction #1: "Page 2 Lines 1-2: CO2 should be subscript"

Response: Corrected.

Technical correction #2: "Page 4 Line 2: m3 should be superscript"

Response: Corrected.

Technical correction #3: "Page 4 Line 10: CO2 should be subscript"

Response: Corrected.

Technical correction #4: "Page 5 Line 5: lenght3 check type error"

Response: Corrected.

Technical correction #5: "Page 6 Line 12: mg-1 should be superscript"

Response: Corrected.

Technical correction #6: "Page 6 Line 23: mg-2 should be superscript"

Response: Corrected.

Technical correction #7: "Pag 10 Line 20: H+ should be superscript"

Response: Corrected.

Technical correction #8: "Page 9 line 17: the word non-lethal could be replaced by sublethal, which is more often used in toxicity studies"

Response: We have changed the terms.

Technical correction #9: "Page 9 line 19: A. regius should be written in italic"

Response: Changed.

Please also note the supplement to this comment:
https://www.biogeosciences-discuss.net/bg-2017-147/bg-2017-147-AC2-

supplement.pdf

Interactive comment on Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-147, 2017.