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 meagre (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, ~0.12 mg kg^{-1} wet weight (ww); and high contamination, ~1.6 mg kg^{-1}ww 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 ± 0.01 mg kg^{-1} dry weight (dw) and 8.02 ± 0.01 mg kg^{-1} 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 CO_2 (co-occuring with Hg contamination) is linked to upregulation of the lysosome-autophagy pathway, which is responsible for removing damaged proteins 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 CO_2 levels used (400 and 1500 µatm), i.e. delta (Δ) CO_2 = 1100 µatm. The presentation rationale follows that used for presenting temperature effects: we used 19 and 23 ºC, i.e. delta (Δ) T = 4 ºC.

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 (~8.00) and seawater temperatures registered at that time of the year (19 ºC), which we used to serve as our control parameters. We have added this information in the text:

"Juvenile *Argyrosomus regius* (n ≃ 100; Fig. 5) (mean ± SD; total weight: 4.26 ± 2.8 g; total length: 6.30 ± 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 ºC). 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 (NH_3/NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) concentrations were daily checked (Colorimetric kits, Aquamerk, Germany), and kept below detectable levels (i.e. $NH_3/NH_4^+ < 0.25$ mg l^{-1}; $NO_2^- < 0.10$ mg l^{-1}; $NO_3^- < 0.2$ 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 (NH_3/NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) concentrations were daily checked (Colourimetric kits,

Aquamerk, Germany), and kept below detectable levels (i.e. NH_3/NH_4^+ < 0.25 mg l^{-1}; NO_2^- < 0.10 mg l^{-1}; NO_3^- < 0.20 mg l^{-1}), and salinity was kept at 35.0 ± 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 pH_T:

"Seawater carbonate system speciation (Table S1) was calculated once every week from $pH_{total\ scale}$ (pH_T) and total alkalinity. pH_T was quantified via a Metrohm pH meter (826 pH mobile, Metrohm, Filderstadt, Germany) connected to a glass electrode (Schott IoLine, SI analytics, ± 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 (C_T), pCO_2 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 underlying 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 CO_2, 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 fed2-3 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 ◦C, 400 pCO2 µatm and contaminated feed (MeHg: 8.02 mg kgÂ´z; HgT: 8.28 mg kgÂ´z). Setup IV should be 19 ◦C, 1500µatm and contaminated feed.”*

Response: We have corrected the characteristics of setup iv): “19 °C, 1500 pCO_2 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 ± 0.025
Certified value		0.410 ± 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 CO_2, 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 $\alpha = 0.05$ in biological statistics and our analysis yielded a $p = 0.116$ for CO_2 * 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 CO_2 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 CO_2 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 ± SE) in *A. regius* muscle driven by an interaction" (Page 2, Line 5)

"Figure 3. a) Catalase (CAT) enzyme activities (mean ± SE) driven by MeHg contamination (Non-contaminated and Contaminated). b) Superoxide dismutase (SOD) activities (mean ± SE) in *A. regius muscle…"* (Page 24, Line 5-6)

"Figure 4. Glutathione S-Transferase (GST) activities (mean ± 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.

Ocean acidification dampens warming and contamination effects on the physiological stress response of a commercially important fish

Eduardo Sampaio[1#*], Ana R Lopes[1#], Sofia Francisco[1], Jose R Paula[1], Marta Pimentel[1], Ana L Maulvault[1,2,3], Tiago Repolho[1], Tiago F Grilo[1], Pedro Pousão-Ferreira[2], António Marques[2,3], Rui Rosa[1]

[1]MARE- Marine Environmental Sciences Centre & Laboratório Marítimo da Guia, Faculdade de Ciências, Universidade de Lisboa, Av. Nossa Senhora do Cabo 939, Cascais 2750-374, Portugal
[2]Divisão de Aquacultura e Valorização (DivAV), Instituto Português do Mar e da Atmosfera (IPMA, I.P.), Av. Brasília, Lisboa 1449-006, Portugal
[3]Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), University of Porto, Rua das Bragas, 289, 4050-123 Porto, Portugal

Correspondence to: Eduardo Sampaio (easampaio@fc.ul.pt)

# equally contributed

Abstract. Increases in carbon dioxide (CO_2) and other greenhouse gases emissions are leading to changes in ocean temperature and carbonate chemistry, the so-called ocean warming and acidification phenomena, respectively. Methylmercury (MeHg) is the most abundant form of mercury (Hg), well-known for its toxic effects on biota and environmental persistency. F~~Despite more than likely co-occurrence in future oceans, the~~uture interactive effects between ~~ofthese~~ contaminants and climate change stressors are still largely unknown, even though such interactions will play a key role in shaping the ecophysiology of marine organisms. Here we assessed organ-dependent Hg accumulation (gills, liver and muscle) within a warming (ΔT = 4 ºC) and acidification (ΔpCO_2 = 1100 µatm) context, and the respective phenotypic responses of molecular chaperone and antioxidant enzymatic machineries, in a commercially important fish (the meagre *Argyrosomus regius*). After 30 days of exposure, although no mortalities were observed in any treatments, Hg concentration was ~~significantly~~ enhanced under warming conditions, ~~significantly more so~~especially in the liver. On the other hand, increased CO_2 decreased Hg accumulation and, despite negative effects prompted as a sole stressor, consistently elicited an ~~antagonistic~~ opposing effect relatively to~~withtemperature~~ warming and contamination on oxidative stress (catalase, superoxide dismutase and glutathione-S-tranferase activities) and heat shock (Hsp70 levels) responses. Together with CO_2-promoted removal of damaged proteins and enzymes, w~~W~~e argue that ~~the mechanistic interactions are grounded on~~ simultaneous increase in ~~excessive~~ hydrogen (H^+) and reactive oxygen species (e.g. O_2^-) ~~free~~ radicals ~~,and subsequent~~is partially compensated through chemical reaction equilibrium balancing. Additional multi-stressor experiments are needed to

understand such biochemical mechanisms and further disentangle interactive (additive, synergistic or antagonistic) stressor effects on fish ecophysiology in the oceans of tomorrow.

1 Introduction

Atmospheric carbon dioxide (CO_2) concentrations have been increasing since the preindustrial era (≃400 CO_2 µatm nowadays), and are expected to reach approximately 1000 CO_2 µatm by the year 2100 (IPCC, 2014). Moreover, ~~Increased CO_2,along~~conjointly with other "greenhouse" gase~~s~~, increased CO_2 has trigger~~ed~~ a continuous rise in mean ocean temperatures ~~due to greenhouse effect,~~(nowadays increased by 0.76 ºC from pre-industrial values), and predictions point to a further ~~a~~ 0.3-4.8 °C ~~global temperature~~ increase ~~between 0.3 °C to 4.8 °C~~ by the end of the century (IPCC, 2014). A~~Simultaneously, a~~tmospheric CO_2 dissolves in the ocean, altering seawater carbonate chemistry. Carbon dioxide uptake increases hydrogen ion (H^+) availability, leading to a concomitant decrease of 0.13-0.42 units in mean ocean pH by the year 2100, i.e. ocean acidification (IPCC, 2014). Due to naturally frequent variations in seawater physicochemical properties (e.g. upwelling events, significant carbon input from river basins), a more accentuated CO_2 input will occur in coastal areas, easily reaching pCO_2 values beyond 1500 µatm (Melzner, 2013). The combined occurrence of ocean warming and acidification imposes ecophysiological challenges to marine organisms, eliciting interactive negative effects on survival, growth and overall physiological fitness (Harvey et al., 2013; Kroeker et al., 2010; Pimentel et al., 2015).

In addition to global warming and ocean acidification, marine biota will also deal with an additional major stressor: contamination. One of the most concerning and persistent metal contaminants is mercury (Hg) and its ubiquitous environmental compound, methylmercury (MeHg) (Korbas et al., 2011). Inorganic mercury is methylated into organic MeHg by bacteria present in the sediment of estuaries and coastal areas (Dijkstra et al., 2013), augmenting Hg bioavailability, bioaccumulation and biomagnification in marine organisms throughout the food web (Campbell et al., 2005; Evers et al., 2011). In teleost fish, MeHg accumulates preferentially in organ tissue, producing site-specific structural and functional damage (Gonzalez et al., 2005), and comprises around 90–95% of total mercury (HgT) in the organism (Burger et al., 2003; Gray et al., 2000). Mercury accumulation can cause deleterious effects, such as physiological distress, i.e. activation of antioxidant and xenobiotic defense (Gonzalez et al., 2005; Mieiro et al., 2010), behavioural and organ functionality impairments (Berntssen et al., 2003; Sampaio et al., 2016) and~~,~~ ultimately~~,~~ mortality (Coccini et al., 2000).

Contaminant uptake and its impacts are potentially shaped by increased temperature or CO_2 and vice-versa (Noyes et al., 2009). Specifically, interactions between temperature and heavy metal contamination influence the physiological tolerance to both stress factors (Sokolova and Lannig, 2008) while exacerbating biological responses (Dorts et al., 2014; Lapointe et al., 2011; Sappal et al., 2014). Consequently, MeHg accumulation is augmented and propagation throughout the food chain is strengthened, until metabolic thresholds are reached (Dijkstra et al., 2013). In parallel, severe acidification (pH < 7)

increases metal availability (Wiener et al., 1990) and toxicity (Han et al., 2014). However, ~~this~~ such effect may be offset by CO_2-linked ~~metabolism~~ decreases~~,leading to lower~~ in mercury accumulation (Sampaio et al., 2016; Schiedek et al., 2007; Wang et al., 2017)~~via feeding (Sampaio et al., 2016; Schiedek et al., 2007)~~. Under environmental stressor exposure, a general deleterious biochemical pathway triggered is the formation of oxygen reactive species (ROS) in the organism's cells. Although there is some proof linking ROS production to hypercapnic scenarios (Pimentel et al., 2015), such is particularly true for increased temperature and mercury contamination (Berntssen et al., 2003; Portner, 2002). Increasing ROS concentrations cause protein damage and lipid peroxidation, i.e. oxidative stress, cascading in augmented malondialdehyde content (MDA), one of the final products of lipid peroxidation (Lesser, 2006). As a physiological defense response, ROS production elicits antioxidant activity in the organism. Specifically, a battery of enzymes is~~are~~ activated to eliminate ROS and prevent MDA build-up: superoxide dismutase (SOD), which converts superoxide (O_2^-) into hydrogen peroxide (H_2O_2); catalase (CAT) which converts H_2O_2 into water (H_2O) and oxygen (O_2); and glutathione S-transferase (GST), which is involved in the protection against xenobiotics and linked to antioxidant defense (Lesser, 2006; Wang et al., 2000). Moreover, tissue-specific heat shock protein~~s~~ (Hsp70) production are also correlated with thermal stress, i.e. high temperatures (Repolho et al., 2014; Rosa et al., 2012, 2014a) and metal contamination (Rajeshkumar and Munuswamy, 2011; Williams et al., 1996). Heat shock proteins help repair, refold and eliminate damaged or denatured proteins, as well as protect and control ROS formation (Sokolova et al., 2011). Given their wide scope, these constituents of the antioxidant enzymatic and protein chaperone machineries are widely used as biomarkers in ecotoxicology to assess fish physiological stress responses (e.g. Anacleto et al., 2014; Fonseca et al., 2011; Rosa et al., 2014b).

Despite the inevitability of marine organisms having to cope with simultaneous effects of ocean warming, acidification and persistent contamination (MeHg), no studies have focused on how the interactive effects between these three stressors will challenge ~~teleost~~ fish ecophysiology. Due to its coastal distribution, the meagre *(Argyrosomus_regius*) is particularly susceptible to MeHg accumulation, especially when they migrate towards ~~the~~ estuaries to spawn (Durrieu et al., 2005). Understanding how this commercially important species will deal with the predicted climate change scenarios may provide valuable information on future stock population conditions and potential impacts on coastal food-webs. Within this context, here we performed a 30-day acclimation experiment to investigate organ-dependent Hg accumulation (gills, liver and muscle) under a warming (ΔT = 4 ºC) and acidification (ΔCO_2 = 1100 µatm) context, as well as the respective phenotypic responses of molecular chaperone (Hsp70) and antioxidant enzymatic (SOD, CAT and GST) machineries, in commercially important fish (*A. regius*). The direct consequences at organism (survival rates and condition index) and cellular (lipid peroxidation, MDA) levels were also evaluated.

2 Material and Methods

2.1 Experimental setup and incubation

Juvenile *Argyrosomus regius* (n ≃100; Fig. 5) (mean ± SD; total weight: 4.26 ± 2.8 g; total length: 6.30 ± 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 ºC). In August 2014, fish were transported to the facilities of Laboratório Marítimo da Guia (LMG, MARE, Faculdade de Ciências, Universidade de Lisboa) ~~in August 2014~~. Fish were randomly placed in twenty-four 50l tanks (n = 3-4 per tank) with individual recirculating aquaculture systems (RAS) equipped with glass wool (physical filtration), bio-balls (Fernando Ribeiro Lda) and protein skimmers (biological filtration, ReefSkimPro 850, TMC Iberia), as well as additional UV disinfection (Vecton 120, TMC Iberia) to maintain superior water quality. Natural seawater was pumped directly from the ocean into an 8 m^3 storage tank, and subsequently filtered (0.35 µm filters, Fernando Ribeiro Lda) and UV-sterilized (Vecton600, TMC Iberia), before pumping into mixing (n = 24) and respective experimental (n = 24, 50 l) tanks/RAS. To prevent fluctuations in environmental parameters, e~~E~~ach 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 (NH_3/NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) concentrations were daily checked (Colourimetric kits, Aquamerk, Germany), and kept below detectable levels (i.e. NH_3/NH_4^+ < 0.25 mg l^{-1}; NO_2^- < 0.10 mg l^{-1}; NO_3^- < 0.20 mg l^{-1}), and ~~Ammonia, nitrate and nitrite were regularly monitored and kept within recommended levels (Aquamerk). s~~Salinity was kept at 35.0 ± 1.0 (V2 Refractometer, TMC Iberia, Portugal). ~~gl^{-1} and thephotoperiod was fixed to 12 h light: 12 h dark.~~ Temperature ~~, salinity~~ and pH (multiparametric probe, Multi3420 SET G, WTW) were ~~daily~~ measured daily, directly in the holding tanks. Photoperiod was fixed at 12 h light : 12 h dark.

As per experimental conditions, temperature in the tanks was down-regulated using chillers (± 0.1 ºC, Frimar, Fernando Ribeiro Lda), and up-regulated by submerged 200 W heaters (V2Therm, TMC Iberia). Seawater carbonate chemistry was altered through CO_2-enriched air input, with pH (8.0 and 7.5) used as proxy measurement. ~~As pH controller, W~~we used a Profilux system (± 0.1, Profilux 3.1N, GHL) as pH controller, connected to each tank by individual pH probes. Within each RAS, pH was down-regulated by injection of the certified CO_2-enriched air (Air Liquide), and up-regulated by injection of atmospheric air. Seawater carbonate system speciation (Table S1) was calculated once every week from $pH_{total\ scale}$ (pH_T) and total alkalinity~~the latter (wavelength = 595 nm,)using a base neutralization by formic acid and a pH sensitive dye (bromophenol blue), following Sarazin et al. (1999).~~ . pH_T was quantified via a Metrohm pH meter (826 pH mobile, Metrohm, Filderstadt, Germany) connected to a glass electrode (Schott IoLine, SI analytics, ± 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 (C_T), pCO_2 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). The non-contaminated and contaminated fish were fed similar diets, differing only on MeHg content. Contaminated diet was fortified with MeHg (inserted in the form of MeHg(II) chloride, CH_3ClHg, 99.8 %, Sigma-Aldrich, solubilized previously in ethanol). Given our dietary option, ecologically relevant MeHg concentrations wasere chosen based on levels (low contamination, ~0.12 mg kg^{-1} wet weight (ww); and high contamination, ~1.6 mg kg^{-1} ww 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 ± 0.01 mg kg^{-1} dry weight (dw) and 8.02 ± 0.01 mg kg^{-1} ~~dry weight (~~dw~~)~~ of MeHg, respectively, ~~The pellet given to the fish allocated to~~in the contaminated treatment had ~~approximately 8.02 ± 0.01 mg kg^{-1} dw of MeHg and 8.28 ± 0.01 mg kg^{-1} dw of HgT~~which were considered to mimickthe concentrations found in the field (see Maulvault et al., 2016, 2017). Feed composition, manufacturing and MeHg spiking processeswere executed as described by ~~(~~Maulvault et al.~~,~~(2016). ~~Given our dietary option, MeHg concentration was chosen based on levels foundin common *A. regius* prey species from contaminated coastal areas(Cardoso et al., 2014; Nunes et al., 2008). An ecologically relevant concentration was chosen, indicated by previous studies on contaminated coastal areas (Nunes et al., 2008). MeHg exposure occurred via feed intake.~~ Fish were fed two to three times a day and total fee~~oo~~d ~~amount~~quantity provided per day was approximately 1% (standard calculation for aquaculture) of animal weight~~.~~ (at the end of 30 days,each fish was given approximately 0.0106 mg of HgT). Selected f~~F~~eed quantity ~~was calculated to~~also minimized food remains, which, in case of existing, were siphoned together with fish faeces ~~one hour~~ after feeding.

After 15 days of lab acclimation (control conditions: 19 ºC, $CO_2 \simeq 400$ µatm), fish were kept during 30 days under crossed-treatments of ocean warming (ΔT = 4 °C), acidification (ΔpH = 0.5 units, i.e. ΔpCO_2 = 1100 µatm) and MeHg contamination (contaminated and non-contaminated) in a full-factorial design, simulating predicted "business-as-usual" scenarios for the year 2100 (IPCC, 2014; Melzner et al., 2013; Schiedek et al., 2007). The experimental setup mimicked the design elaborated by Cornwall and Hurd (Fig. 3d, 2015). More specifically, the setup was divided in eight treatments (n = 3 tanks per treatment): i) 19 °C, 400 pCO_2 µatm (control conditions) and non-contaminated feed (MeHg: 0.06 mg kg^{-1}; HgT: 0.07 mg kg^{-1}), ii) 19 °C, 400 pCO_2 µatm and contaminated feed (MeHg: 8.02 mg kg^{-1}; HgT: 8.28 mg kg^{-1}), iii) 19 °C, 1500 pCO_2 µatm (control temperature and hypercapnic scenario) and non-contaminated feed (MeHg: 0.06 mg kg^{-1}; HgT: 0.07 mg kg^{-1}), iv) 19 °C, 1500 pCO_2 µatm and contaminated feed (MeHg: 8.02 mg kg^{-1}; HgT: 8.28 mg kg^{-1}); v) 23 °C, 400 pCO_2 µatm (warming and normocapnic scenario) and non-contaminated feed (MeHg: 0.06 mg kg^{-1}; HgT: 0.07 mg kg^{-1}); vi) 23 °C, 400 pCO_2 µatm and contaminated feed (MeHg: 8.02 mg kg^{-1}; HgT: 8.28 mg kg^{-1}); vii) 23 °C, 1500 pCO_2 µatm (warming and hypercapnic scenario) and non-contaminated feed (MeHg: 0.06 mg kg^{-1}; HgT: 0.07 mg kg^{-1}); and viii) 23 °C, 1500 pCO_2 µatm and contaminated feed (MeHg: 8.02 mg kg^{-1}; HgT: 8.28 mg kg^{-1}).

Survival rates were monitored throughout the experiment, and after 30 days fish were measured (total length) and weighed. Health status was assessed through the widely used Fulton's condition factor K (n = 6-8 per treatment), described by the following formula: $K = 100 \times (Weight / Length^3)$. Individuals were anesthetized with MS-222 and euthanized by swift spinal cord severing. Sample tissues of three organs (muscle, liver and gills) were harvested for further analysis.

2.2 Total mercury and Methylmercury accumulation

Methylmercury extraction from samples (fish and different feeds, n = 3-6) was performed as described by Scerbo and Barghigiani (1998), i.e. freeze-dried samples (~200 mg) were hydrolyzed in 10 ml of hydrobromic acid (47 % w/w, Merck), following addition of 35 ml toluene (99.8 % w/w, Merck) to allow MeHg extraction and removal with 6 ml cysteine solution (1 % L-cysteinium chloride in 12.5 % anhydrous sodium sulfate and 0.775 % sodium acetate, Merck). Afterwards, HgT (all samples) and MeHg (feed samples) were determined ~~in all samples~~ (10-15 mg for solids or 100-200 µl for liquids) by atomic absorption spectrometry (AAS), following EPA (2007) by means of an automatic Hg analyser (AMA 254, LECO, USA) with a detection threshold of 0.005 mg kg^{-1}~~, wet weight (~~ww~~)~~. Mercury concentrations were calculated through linear calibration (using > 5 standard concentrations), with a Hg(II) nitrate standard solution (1000 mg l^{-1}, Merck) dissolved in nitric acid (0.5 mol l^{-1}, Merck). Accuracy was checked by also analyzing certified reference material DORM-4, and framing results obtained within the certified range of values (Table S2). A minimum of three measurements were performed per sample. Blanks were always tested in the same conditions as the samples and measurements were taken in triplicate. All laboratory ware was previously cleaned using~~ with~~ nitric acid (20 % v/v) for 24h and ultrapure water, in that order. All standards and reagents were of analytical (pro analysis) or superior grade.

2.3 Enzymatic assays

2.3.1Preparation of tissue extracts

Muscle, liver and gill~~s~~ samples (n = 4-6 per tank) were homogenized (Ultra-Turrax, Staufen, Germany) in accordance to body mass of each sample in homogenization buffer, 300 mg tissue per 1 ml phosphate buffered saline solution (PBS, pH 7.4): 0.14 M NaCl, 2.7 mMKCl, 8.1 mM Na_2HP0_4, 1.47 mM KH_2P0_4. H~~Posteriorly, h~~omogenates were centrifuged (20 min at 14000 rpm at 4 °C) and antioxidant enzyme activities, as well as lipid peroxidation and heat shock response concentrations~~ were~~ quantified in the supernatant fraction. All enzyme assays were tested with commercial enzymes obtained from Sigma-Aldrich (St. Louis, USA), and each sample was run in triplicate (technical replicates). The enzyme results were normalized with total protein content following the Bradford method (Bradford, 1976).

2.3.2 Lipid peroxides assay (malondialdehyde concentration)

As an end-product of oxidative stress, malondialdehyde (MDA) concentration was used as a proxy to assess extent of lipid peroxidation in the muscle. We used the thiobarbituric acid reactive substances (TBARS) protocol described by Uchiyama and Mihara, (1978). A total of 10µl of each sample were added to 45 µl of 50 mM monobasic sodium phosphate buffer, followed by addition of 12.5 µl of sodium dodecyl sulfate (8.1%), 93.5 µl of trichloroacetic acid (20%, pH = 3.5) and 93.5 µl of thiobarbituric acid (1%) to each microtube. Then, 50.5 µl of ultrapure water were added to this mixture and placed in a vortex for 30 s. A needle was used to puncture the lids and microtubes were incubated in boiling water (10 min) followed by ice cooling. Subsequently, 62.5 µl of ultrapure water and 312.5 µl of n-butanol pyridine (15:1, v/v) (Sigma-Aldrich, Hamburg, Germany) were added and microtubes centrifuged (5000 x g; 5 min.). 150 µl of the supernatant's reaction were introduced into a 96-well microplate in duplicate and absorbance was read at 530 nm. Lipid peroxides (i.e., MDA concentration) were determined using malondialdehyde (dimethylacetal) (MDA) (Merck, Switzerland) standards in an eight-point calibration curve (0–0.3 µM TBARS). Results were expressed in relation to the sample total protein (nmol mg^{-1} total protein).

2.3.3 Catalase (CAT) activity

Catalase activity in the muscle was assessed through an adaptation of the method described by Johansson and Borg (1988). In this assay, 20 µl of each sample, 100 µl of 100 mM potassium phosphate and 30 µl of methanol were added to a 96-well microplate, which was promptly shaken and incubated for 20 minutes. Afterwards, 30 µl of potassium hydroxide (10 M KOH) and 30 µl of purpald (34.2 mM in 0.5 M HCl) were added to each well, and the plate shaken and incubated for another 10 minutes. Subsequently, 10 µl of potassium periodate (65.2 mM in 0.5 M KOH) was added to each well and a final incubation was performed for 5 minutes. Using a microplate reader (Asys UVM 340, Biochrom, USA), enzymatic activity was determined spectrophotometrically at 540 nm. Formaldehyde concentration of the samples was calculated based on a calibration curve (from 0 to 75 µM formaldehyde), followed by the calculation of CAT activity for each sample, where one unit of CAT is defined as the amount that will cause the formation of 1.0 nmol of formaldehyde per minute at 25 ºC. The results are expressed in relation to total protein content (nmol min mg^{-2} protein).

2.3.4 Superoxide Dismutase (SOD) activity

SOD activity in the muscle was determined following the nitro blue tetrazolium (NBT) method adapted from Sun et al. (1988). Superoxide radicals (O_2^-) are generated by xanthine oxidation, and simultaneous reduction of NBT to formazan. SOD competes with NBT for the dismutation of O_2^- into hydrogen peroxide (H_2O_2) and molecular oxygen, and this is used to determine enzyme activity. Briefly, the assay was performed using a 96-well microplate (Nunc-Roskilde), adding to each well 200 µl of 50 mM phosphate buffer (pH 8.0) (Sigma-Aldrich), 10 µl of 3 mM EDTA (Riedel-de Haën, Seelze,

Germany), 10 µl of 3 mM xanthine (Sigma-Aldrich), 10 µl of 0.75 mM NBT (Sigma-Aldrich) and 10 µl of SOD standard or sample. Reaction began by adding 10µl of 100 mU xanthine-oxidase (XOD, Sigma-Aldrich) and absorbance (560 nm) was recorded every 5 minutes for 25 minutes, using a plate reader (Asys UVM 340, Biochrom, USA). SOD from bovine erythrocytes (Sigma-Aldrich) was used as standard and positive control, and a negative control included all components except SOD or sample. The latter yielded a maximum threshold in absorbance, which allowed the assessment of inhibition percentage per minute (averaged from 25 minutes), which is caused by SOD activity. Thus, SOD activity percentage was expressed in % inhibition mg^{-1} of total protein.

2.3.5 Gluthationse S-Tranferase (GST) activity

GST ~~total~~ activity 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). 1-Chloro-2,4-dinitrobenzene (CDNB) is used as substrate and, upon conjugation of the thiol group of glutathione to the CDNB, absorbance is increased and enzymatic activity can be determined spectrophotometrically. The assay included 200 mM L-glutathione (reduced), 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) solution and Dulbecco's PBS. Equine liver GST (Sigma-Aldrich) was used as positive control to validate the assay. 180 µl of substrate solution were added to 20 µl sample in each well of a 96-well microplate (Nunc-Roskilde) and 340 nm absorbance was registered every minute during 6 minutes, through a plate reader (Asys UVM 340, Biochrom, USA). Finally, GST activity was calculated using a molar extinction coefficient for CDNB of 5.3 εmM (Sigma Technical Bulletin, CS0410), as follows: GST activity = (ΔA340min / 0.0053) x (Total volume / Sample volume) x dilution factor. Results were expressed in relation to total protein of the sample (nmol min mg^{-2} total protein).

2.3.6 Heat shock proteins

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). 10 µl of the supernatant was diluted in 990µl of PBS and 50 µl of that sample were added to a 96-well microplates (Microloan 600, Greiner) and allowed to incubate overnight at 4 °C. On the next day, microplates were washed (three times) in 0.05 % PBS-Tween-20. 100µl of blocking solution (1 % bovine serum albumin (BSA) Sigma-Aldrich) were added to each well and left to incubate for 2 h at room temperature. After washing the 96-well plates, we introduced 50 µl of 5 µg ml^{-1} primary antibody (anti-Hsp70/Hsc70, Acris, San Diego, CA, USA),and again left incubating overnight at 4 °C. According to manufacturer details, the primary antibody Hsp70/Hsc70 (AM12032PU-N) possesses broad range reactivity, e.g. in varied fish species, making it suitable for our analysis. On the next day, the non-linked antibody was removed by washing the microplates, and 50 µl of 1 µg ml^{-1} of the secondary antibody, antimouse IgC, Fab specific, alkaline phosphatase conjugate (Sigma-Aldrich) were added and incubated for 2 h at room temperature. After three additional washes, 100 µl of substrate (SIGMA FASTTM p-Nitrophenyl Phosphate Tablets, Sigma-

Aldrich) was added to each well and incubated 10-30 minutes at room temperature. Stop solution (50 µl; 3 N NaOH) was added in each well, and absorbance was read at 405 nm in a 96-well microplate reader (Asys UVM 340, Biochrom, USA). The amount of Hsp70/Hsc70 present in the samples was calculated from an absorbance/concentration calibration curve based on serial dilutions of purified Hsp70 active protein (Acris), ranging from 0 to 2000 ng ml^{-1}. Results were expressed in relation to the sample total protein (ng mg^{-1}total protein).

2.4 Statistics

All statistical analysis were performed on R Studio (R Development Core Team, 2016). We used Generalized Linear Models (GLM) analysis to infer significant differences between sampled groups (see R script provided in the Supplemental Data for a step-by-step protocol). Mix models, e.g. tank as random factor, were ruled unnecessary as previous analysis (using 'lme4' and 'nlme' packages) showed no significant differences between tanks, within each group treatment, for all variables used. 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. Data was fitted using gaussian family models, and model residuals were checked for homogeneity of variances, independence and leverage were used to perform model validation. When assumptions were not met, we turned to gamma family models to fit our data, and model validation was assessed following the same procedure. Temperature (T, 2 levels: 19 ºC, 23 ºC) CO_2 (CO_2, 2 levels: 400 µatm, 1500 µatm), MeHg exposure (MeHg, 2 levels: Non-contaminated, 0.06 mg kg^{-1}; Contaminated, 8.02 mg kg^{-1}) and organ tissue sampled (Tissue, 3 levels: Muscle, Gills, Liver) were generally used as explanatory variables or factors, according to each specific dependent variable.

3 Results

After 30 days of exposure, no mortalities were registered in any treatment. Fulton condition (K) did not show any significant differences between treatments (MeHg, $p > 0.05$, GLM analysis in Table 1). Significant differences were found in total mercury concentrations between contaminated and non-contaminated scenarios (GLM analysis, $t = 9.079$, $p < 0.001$, see Supplemental Data) and also between tissues analyzed (ANOVA F test, $F = 14.015$, $p < 0.001$, see Supplemental Data). 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). Within each tissue, temperature and CO_2 interacted significantly (T x CO_2, , $p < 0.001$ for all tissues, GLM analysis in Table 2, Figure 1b-d) affecting MeHg accumulation. In other words, temperature increased Hg accumulation and such effect was counter-balanced by elevated CO_2.

Subsequently, lipid peroxidation and oxidative stress were measured in the muscle tissue. A significant antagonistic effect was detected between increasing temperature and MeHg contamination on MDA build-up (T x MeHg, $p < 0.05$, GLM analysis in Table 3, Figure 2). Isolated stressors increased MDA production, however this effect was annulled when both stressors were present. Regarding the antioxidant enzyme machinery, CAT activity was positively affected by MeHg contamination (MeHg, $p < 0.05$, GLM analysis in Table 4, Figure 3a). On the other hand, elevated CO_2 increased SOD activity as a single stressor; yet, when combined with warming (T x CO_2, $p < 0.001$, GLM analysis in Table 4, Figure 3b), the effect was reversed. GST activity was modelled by two interactions between CO_2 and temperature (T x CO_2, $p < 0.01$, GLM analysis in Table 4, Figure 4a), and between CO_2 and MeHg contamination (CO_2 x MeHg, $p < 0.01$, GLM analysis in Table 4, Figure 4b). Not reporting strong effects as a sole stressor, increased CO_2 inhibited GST activity when combined with warming (Figure 4a) or MeHg contamination (Figure 4b).

Concerning heat shock response, Hsp70 production varied between the analyzed organs (ANOVA F test, $F = 11.732$, $p < 0.001$, see Supplemental Data), reporting higher concentrations in the liver and lower in the gills (liver > muscle > gills; see GLM analysis in Table 5 for p values, Figure 5a). Within the gills, Hsp70 concentration was positively affected by MeHg contamination (Gills, $p < 0.05$, GLM analysis in Table 5, Figure 5b). On the other hand, in the muscle, temperature and CO_2 modulated Hsp70 production (T x CO_2, $p < 0.001$, GLM analysis in Table 5, Figure 5c). While isolated, elevated CO_2 increased Hsp70 production, but under simultaneous warming, heat shock response was significantly decreased. Concomitantly, temperature-driven Hsp70 increase was also dampened by hypercapnia. Similarly, in the liver, Hsp70 concentration MeHg contamination increased Hsp70 production, but this effect was countered by increased CO_2 (CO_2 x MeHg, $p < 0.01$, GLM analysis in Table 5, Figure 5b).

4 Discussion

4.1 Non-lethal preferential accumulation

The present study showed that Hg contamination, ocean warming and acidification interactively affected fish physiology~~ical condition~~ at ~~non-lethal~~sublethal levels, i.e. zero mortality and also no effects on Fulton condition were~~as~~ registered. ~~However, our AIC-chosen best model indicated that mercury may diminish organism Fulton condition, which is in agreement with previous results obtained in river fish populations (Pyle et al., 2005).~~ The fact that the meagre (*A. regius*) is a very resilient species and easily adapts to environmental alterations (Monfort, 2010) may explain the absence of deleterious effects at an organism level,after 30 days of exposure ~~at an organism level~~.

Affinity for metal accumulation varied between fish tissues with increasing Hg accumulation as follows: muscle < gills < liver. These results are supported by previous reports on mercury tissue preferential accumulation. The muscle is an organ tissue generally characterized for its low metal affinity (Jezierska and Witeska, 2006) compared to~~, e.g.~~ the liver, where

metals accumulate at higher levels, due to its key role in metal accumulation and detoxification (Gbem et al., 2001; Wagner and Boman, 2003). Furthermore, as a result of increased blood supply, gills are organs likewise known to possess higher Hg affinity than the muscle (Jezierska and Witeska, 2006; Vergilio et al., 2012).

4.2 Environmental influence on mercury accumulation

Mercury accumulation in fish is known to depend on the water physicochemical properties (e.g. temperature, pH, alkalinity) (Harris and Bodaly, 1998; Ponce and Bloom, 1991; Wren et al., 1991). Indeed, we also showed a consistent increase in Hg accumulation under the warming scenario. However, when both temperature and CO_2 stressors were present, Hg accumulation was ~~notoriously~~ decreased. Temperature increases Hg bioaccumulation in fish due to enhanced metabolism and consequent higher intake of MeHg-contaminated prey (Dijkstra et al., 2013; MacLeod and Pessah, 1973). Despite previous evidence that lowered pH (< 7.0 units) increases Hg accumulation in freshwater fish (Haines et al., 1992; Ponce and Bloom, 1991), the current findings do not reflect this pattern, arguably due to the magnitude of pH decrease (here we used pH 7.5). Instead, our results support recent studies ~~other reports~~ demonstrating that ~~fish exposed to~~ hypercapnia dampens Hg accumulation in marine organisms (Li et al., 2017; Sampaio et al., 2016; Wang et al., 2017). ~~may display metabolicdecrease~~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 CO_2, enabling better animal fitness which may potentially reduceHg accumulation and toxicity (Wang et al., 2017). In addition, ~~due to prioritization of CO_2 excretory physiological processes(Perry et al., 1988; Sampaio et al., 2016)~~t~~. Thus, t~~aking ~~also~~ into account that the occurrence of both ~~stressors~~ warming and acidification ~~lowers~~ changes physiological ~~(and consequently metabolic)~~ thresholds (Christensen et al., 2011; Harley et al., 2006; Rosa et al., 2013; Rosa and Seibel, 2008)~~(Harvey et al., 2013; Rosa et al., 2013)~~, ~~it is likely that~~ a ~~certain~~ degree of metabolic depression ~~arrest~~ may also play~~ed~~ a ~~key~~ role on decreasing HgT ~~concentration~~ accumulation ~~decrease~~(Dijkstra et al., 2013; Sampaio et al., 2016). From a consumer perspective, our study showed that the ~~CO_2~~counteracting CO_2 effect (hampering warming-stimulated Hg accumulation) was consistent in the muscle, the main tissue ingested by human population. Since thisis the ~~main fish tissue consumedby human populations worldwide~~most relevant tissue for commercialization, such results constitute an important finding in the area of seafood safety, worthy of further research.

4.3 Oxidative stress under a multi-stressor environment

Exposure to MeHg contamination, ocean warming and acidification potentiated significant changes in meagre physiology. As expected, lipid peroxidation and consequent MDA build-up was higher under MeHg contamination (Berntssen et al., 2003; Vieira et al., 2009). The fact that contamination and warming per se elicited only small MDA build-up, is likely due to the fact that *A. regius* is a highly resilient estuarine species, i.e. great tolerance to environmental stressors (Monfort, 2010). Moreover, t~~T~~o 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). While it is worth mentioning that increased CO_2 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. However when combined with other stressors, elevated CO_2 antagonized the co-ocurring stressor's effect (i.e. contamination and/or warming). Increased CO_2 (co-occuring with Hg contamination) may elicit the up-regulation of the lysosome-autophagy pathway, which is responsible for removing damaged proteins and organelles, effectively reducing oxidative stress (Wang et al., 2017). This potential mechanism may contribute to alleviate not only Hg induced stress, but also warming-related oxidative stress. We also argue that ~~such~~ this antagonistic relation can be partially explained by ~~the dramatic~~a CO_2-related increase of H^+ ion concentrations in the blood and cellular surroundings, counterbalanced by bicarbonate increase (acid-base compensation) to normalize pH levels ~~stemming from increased CO_2~~(Heuer and Grosell, 2014; Michaelidis et al., 2007)~~(Michaelidis et al., 2007)~~. By itself, the presence of excessive H^+ ions activates free radical neutralizing defenses (Tiedke et al., 2013), which is in line with the present findings when hypercapnia was the sole stressor. H~~h~~owever, the production of O_2^- and further complementary ROS free radicals (e.g. OH^-) by other stressors may result in facilitated H_2O and H_2O_2 formation, due to chemical reactions balancing equilibrium (e.g. $H^+ + OH^- \rightleftharpoons H_2O$), thus eliminating free radicals and decreasing activity of antioxidant enzymes to basal standards.

4.4 Protein chaperone functioning under a multi-stressor environment

Hsp70 response was tissue-dependent, showing a pattern similar to HgT tissue preferential accumulation (see first section). Higher liver expression is not unexpected given the fact that this organ plays a key role in metal accumulation and detoxification (Gbem et al., 2001; Wagner and Boman, 2003). More importantly, as observed in antioxidant stress enzymatic machinery, hypercapnia revealed the same antagonistic relationship with other stressor's effects: increased CO_2 down-regulated heat shock response in the livers of contaminated fish and in the muscle of fish under warming. As such, this study confirms that Hsp70 expression is closely correlated with other forms of antioxidant response, such as CAT, SOD and GST (Iwama et al., 1998; Rosa et al., 2012, 2014a). More so than for oxidative stress, the enhanced removal of damaged proteins and enzymes indirectly promoted by increased CO_2 (via up-regulated lysosome-autophagy) may have especially contributed to subside protein chaperone production. G~~Moreover, g~~iven that Hsp70 production can also be stimulated by ~~extreme~~ high

ionic (e.g. H^+) concentrations (Feder and Hofmann, 1999), we ~~speculate that the~~reason that the same additional mechanism by which hypercapnia potentially modulates ~~s~~oxidative stress can be applied for heat shock response~~ expression is likely similar to oxidative stress enzymatic machinery modulation~~. Enhanced CO_2 leads to increased H^+ concentration triggering physiological stress responses, while the facilitated conversion of free ions and radicals (H^+ and O-associated molecules) into H_2O and H_2O_2 leads to reduced stress input by warming, contamination (and hypercapnia itself).

5 Conclusions

In this study, we ~~verified~~observed that sublethal MeHg contamination is organ selective (accumulating to higher levels in the liver) and found that future abiotic conditions modulate its accumulation throughout the organism. In general, warming conditions enhanced MeHg accumulation but CO_2-linked ~~metabolic reductions~~impacts countered this effect. ~~Moreover~~In fact, despite negative effects prompted as a sole stressor, acidification consistently elicited antagonistic responses to temperature and contamination effects on oxidative stress (including ~~ and~~ heat shock response)~~s~~, which may be explained by stimulated removal of damaged proteins and organelles (Wang et al., 2017). ~~Thus~~Moreover, we also argue that the mechanistic interactions found are coadjuvanted~~underpinned~~ by the coinciding increase of ~~excessive~~ hydrogen (H^+) and radical reactive oxygen species (e.g. O_2^-, OH^-), which subsequently nullify each other due to the spontaneous equilibrium of chemical reactions (e.g. $H^+ + OH^- \rightleftharpoons H_2O$).

In the future, it is important to deepen our understanding on this mechanism and evaluate if this antagonistic relationship is conservative throughout other less-resilient species (e.g. non-estuarine ones). Further knowledge on climate change and contamination impacts on fish ecophysiology (and biochemical stress-coping mechanisms) will help towards better comprehension of~~rehend the~~ future fish stocks' health condition and tissue-dependent contaminant accumulation,~~of coastal fish populations and~~ consequently forecasting socio-ecological consequences in the oceans of tomorrow. Another pertinent knowledge gap that has been scarcely addressed is how oxidative stress and lipid peroxidation modify the nutritional value and general palatability of seafood, particularly fish. Thus, further multi-stressor studies on seafood safety and biochemical changes should be performed with the intent of helping stakeholders and regulatory authorities define future consumption recommendations and legislation.

6 Code availability

R code used in the analysis is available as Supplemental material.

7 Data availability

The full dataset is made available as Supplemental material.

8Competing interests

The authors declare no conflict of interest.

9 Author contributions

ES, ARL, SF, AM, PP and RR designed the study. JRP, MP, TR and TFG assisted during the experiment and sampling. AL and SF quantified HgT accumulation. ARL, SF, MP and JRP quantified the enzymes. ES, TR, TFG and RR performed the statistical analysis. ES and ARL wrote the paper, for which all authors contributed with discussion and earlier drafts.

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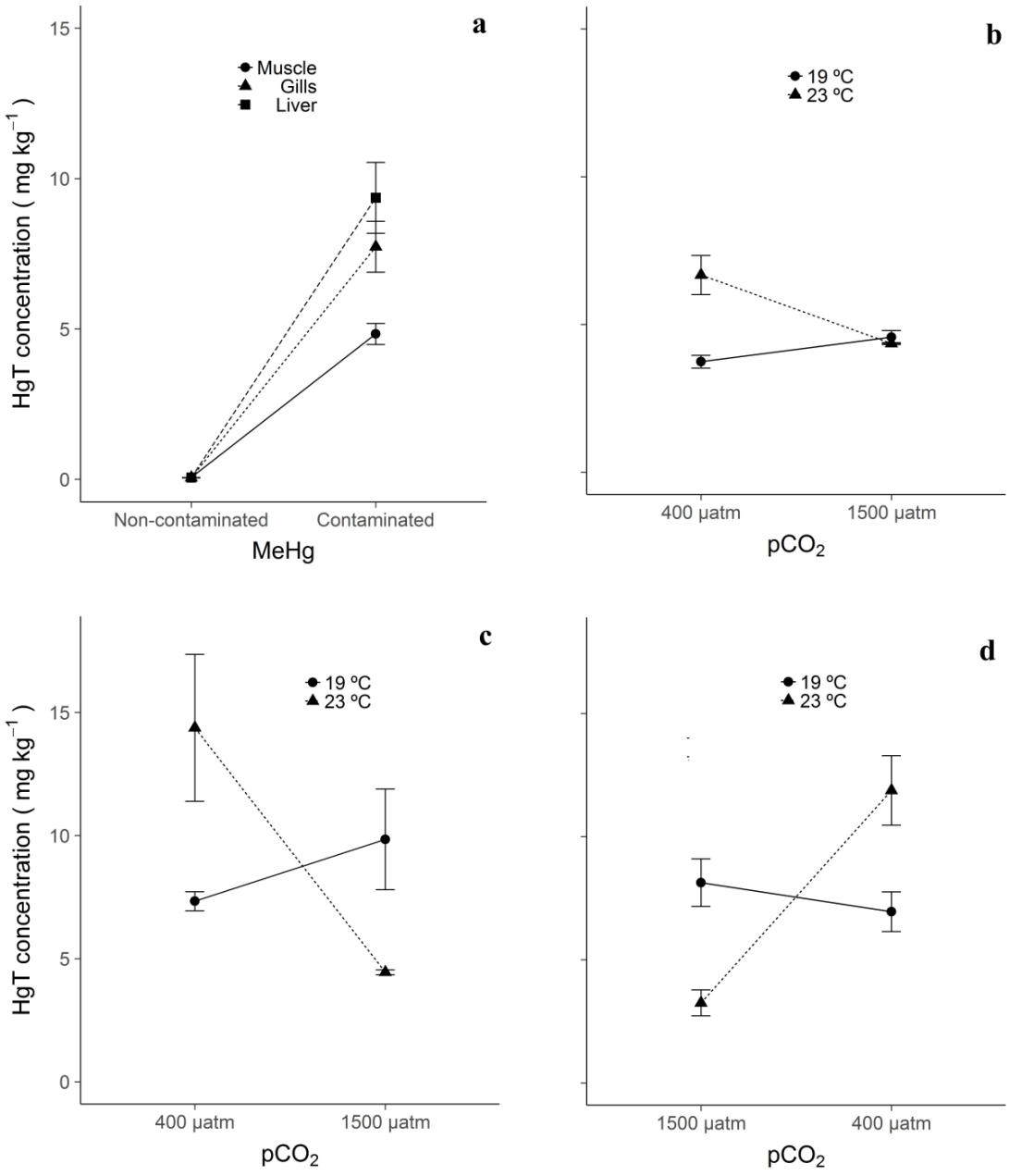
a
Muscle
Gills
Liver
HgT concentration (mg kg^{-1})
15
10
5
0
Non-contaminated
Contaminated
MeHg
b
19 ºC
23 ºC
400 µatm
1500 µatm
pCO$_2$
c
19 ºC
23 ºC
HgT concentration (mg kg^{-1})
15
10
5
0
400 µatm
1500 µatm
pCO$_2$
d
19 ºC
23 ºC
1500 µatm
400 µatm
pCO$_2$

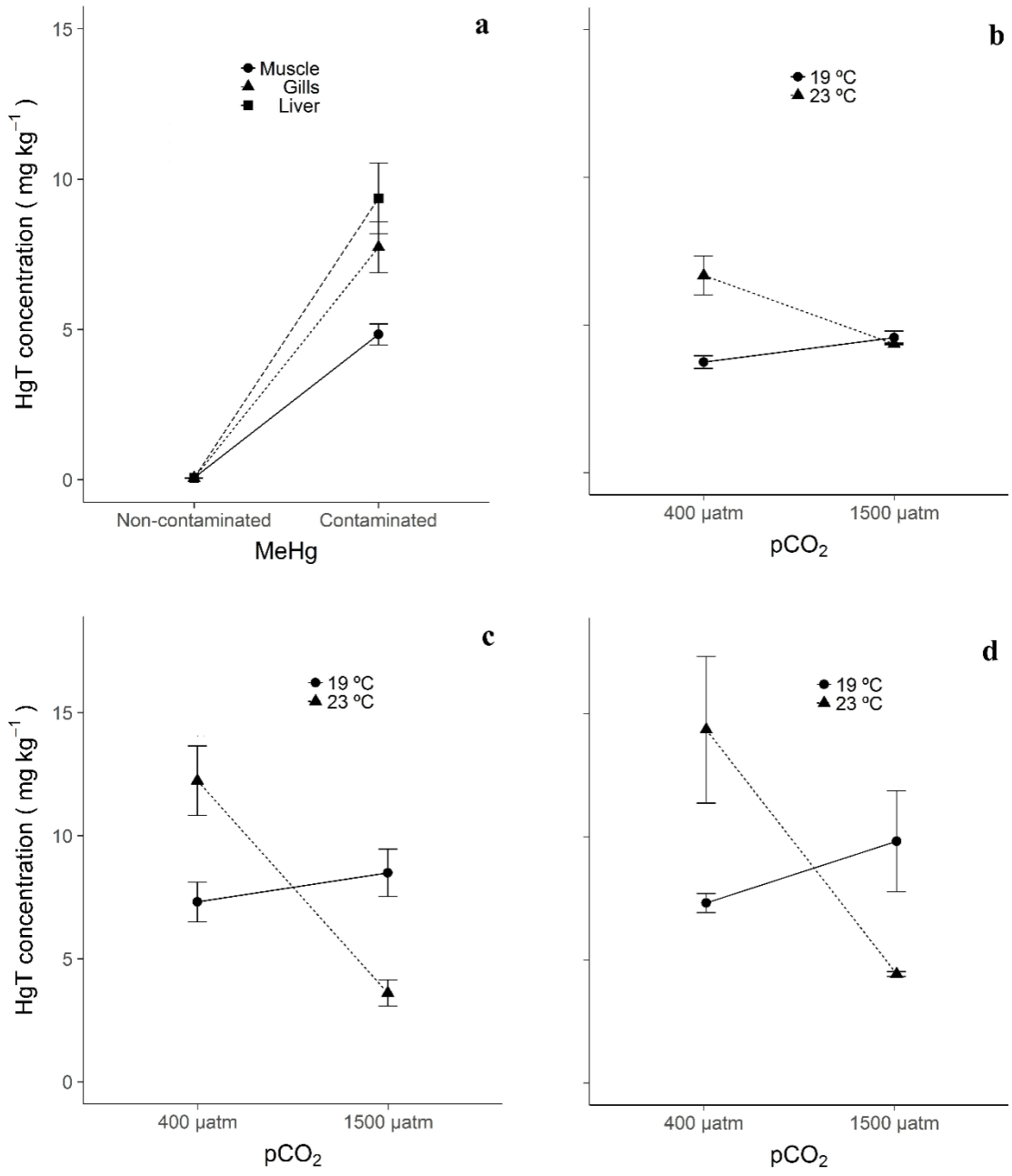

Figure 1. Total mercury (HgT) accumulation (mean ± SE) in *A. regius*: a) Differences among tissues (muscle, gills and liver); and shaped by interactions between temperature (19 and 23 °C) and CO_2 (400 and 1500 µatm) within b) muscle, c) gills and d) liver, respectively. Graphs were plotted according to significant factors yielded by GLM analysis described in Table 1 and 2, respectively.

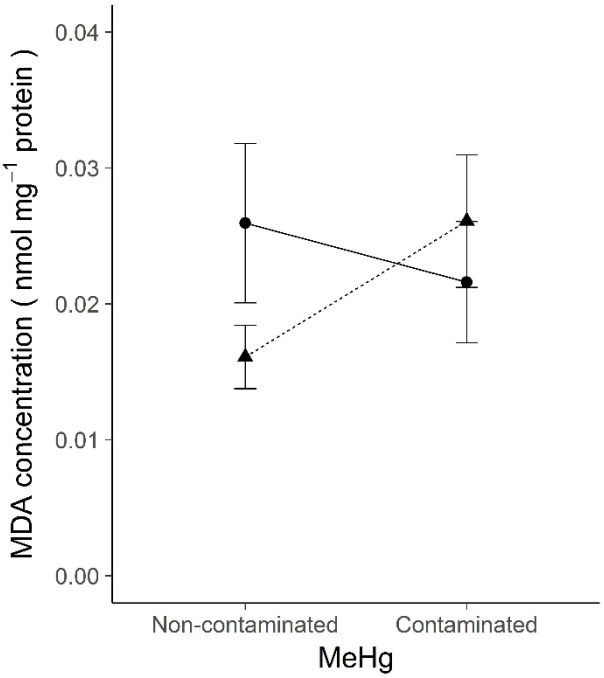

Figure 2. Malondialdehyde (MDA) build-up concentrations (mean ± SE) in *A. regius* muscle driven by an interaction between MeHg contamination (Non-contaminated and contaminated) and temperature (19 and 23 ºC). Graphs were plotted according to significant factors yielded by GLM analysis described in Table 3 and 4, respectively.

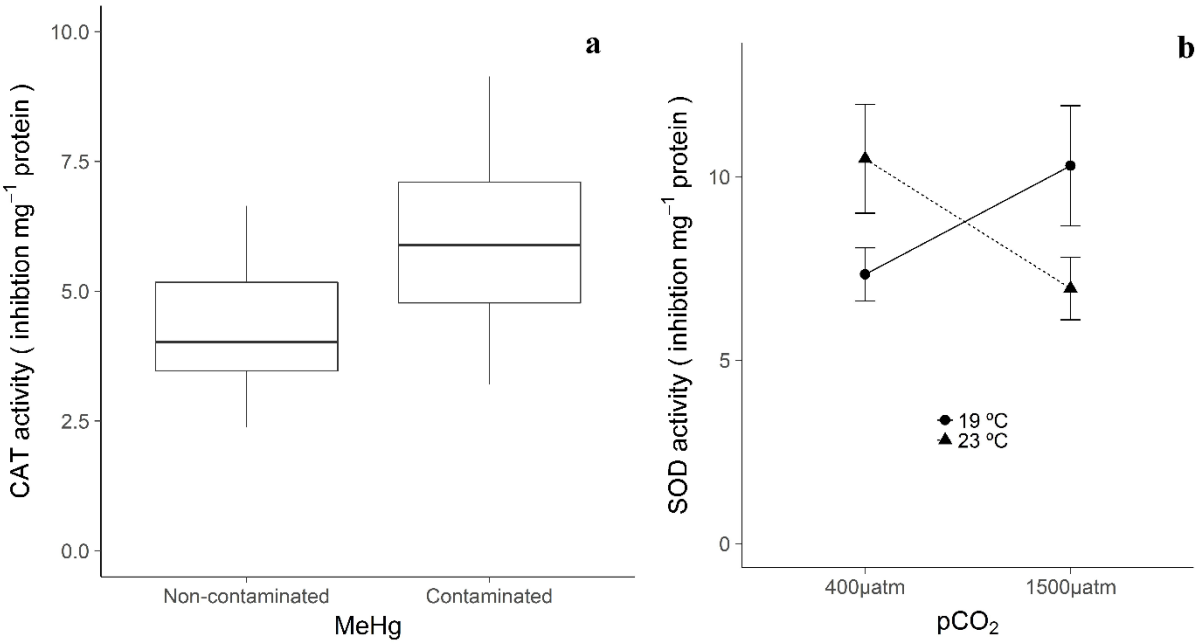

Figure 3. a) Catalase (CAT) enzyme activities (mean ± SE) driven by MeHg contamination (Non-contaminated and Contaminated). b) Superoxide dismutase (SOD) activities (mean ± SE) in *A. regius* muscle driven by an interaction temperature (19 and 23 ºC) and CO_2 (400 and 1500 µatm). Graphs were plotted according to significant factors yielded by GLM analysis described in Table 4.

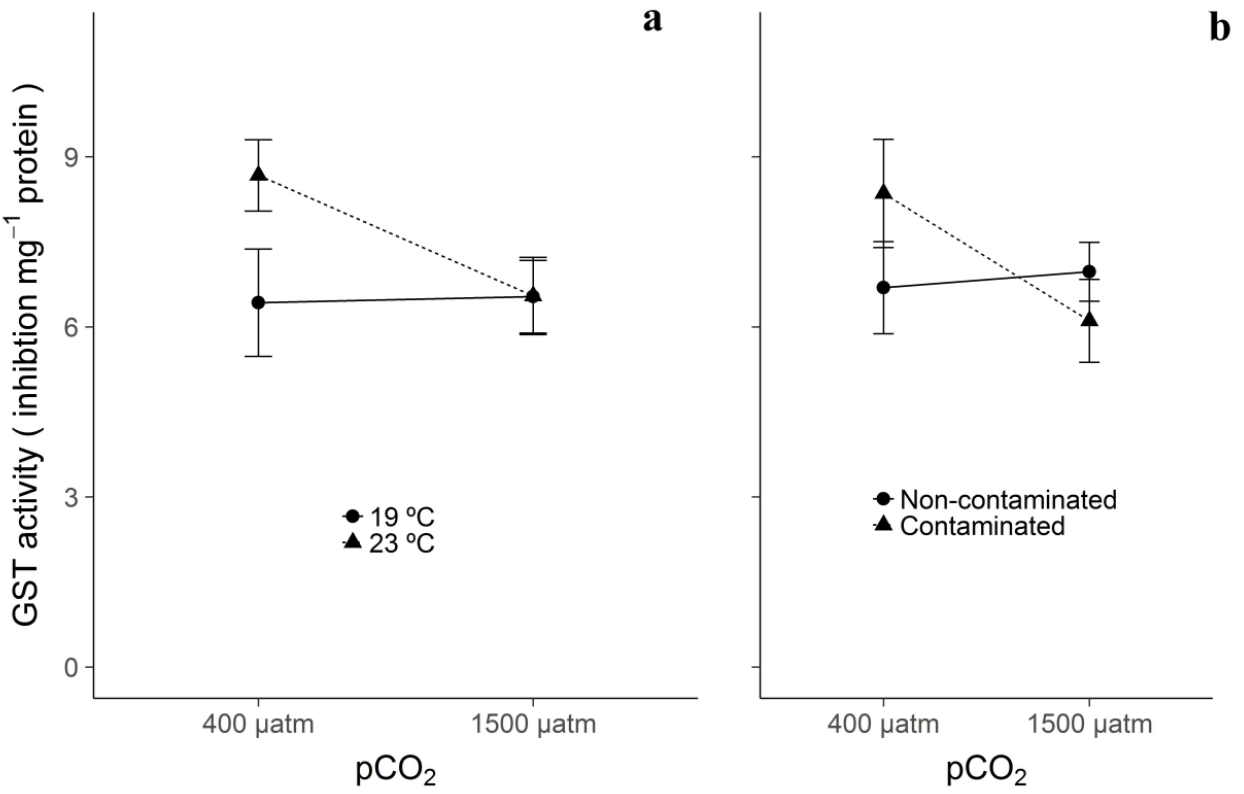

Figure 4. Glutathione S-Transferase (GST) activities (mean ± SE) in *A. regius* muscle driven by: a) an interaction between temperature (19 and 23 ºC) and CO_2 (400 and 1500 µatm); and b) an interaction between MeHg contamination (Non-contaminated and contaminated) and CO_2 (400 and 1500 µatm). Graphs were plotted according to significant factors yielded by GLM analysis (triple interaction) described in Table 4.

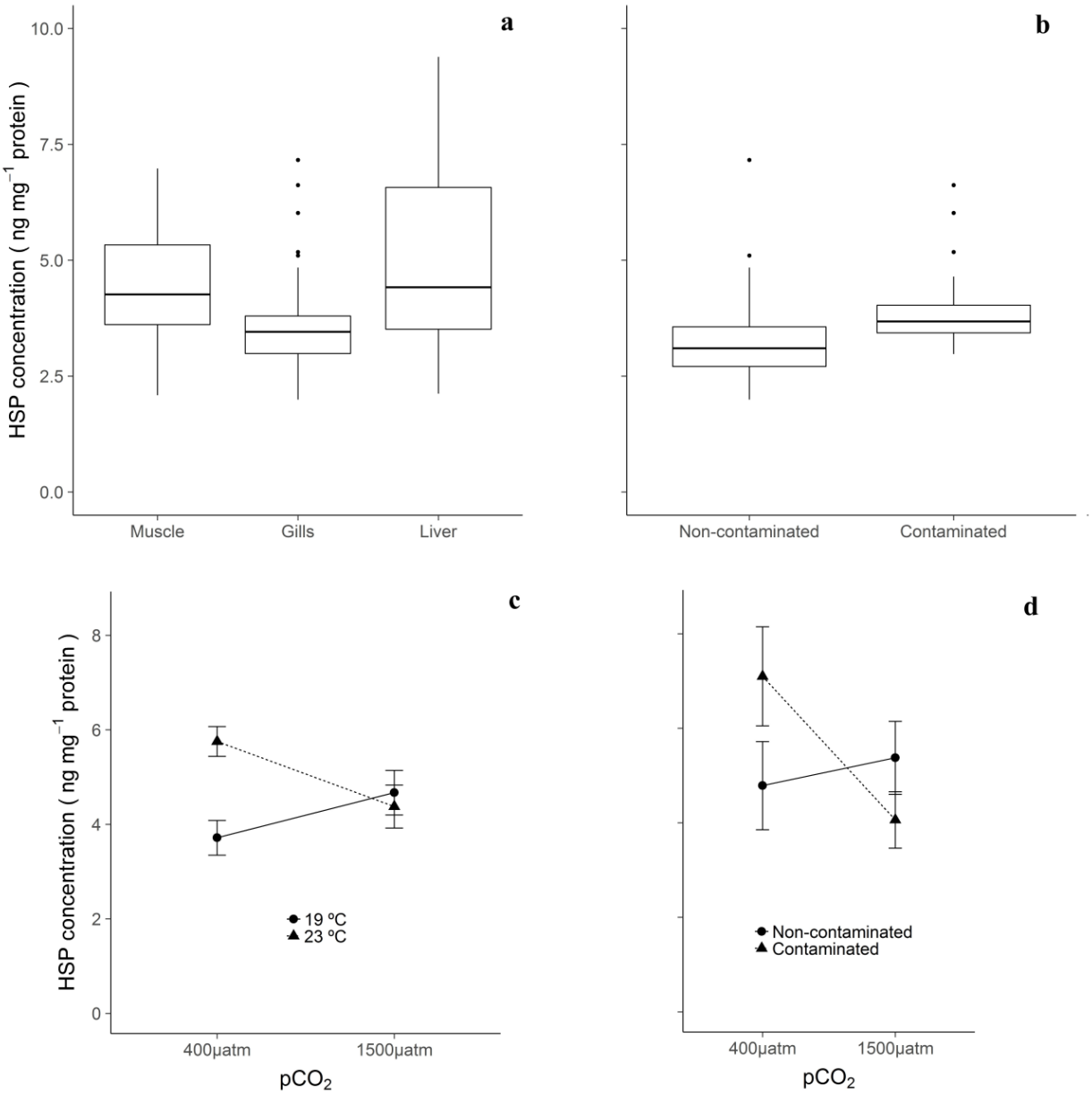

Figure 5. Heat shock protein70 (Hsp70) concentrations (mean ± SE) in *A. regius*: a) tissues; b) in the gills shaped by MeHg contamination (Non-contaminated and Contaminated) and CO_2 (400 and 1500 µatm); in the c) muscle shaped by an interaction between temperature (19 and 23 ºC) and CO_2 (400 and 1500 µatm); and in the d) liver shaped by an interaction between MeHg contamination (Non-contaminated and Contaminated) and CO_2 (400 and 1500 µatm). Graphs were plotted according to significant factors yielded by GLM analysis described in Table 5.

GLM: Fulton's K in function of MeHg

	Est	Std Error	t value	p value
(Intercept)	1.602	0.041	39.09	< **0.001**
MeHg	-0.072	0.057	0.057	0.213

Family = Gaussian AIC = -8.6

GLM: HgT in function of MeHg * Tissues

	Est	Std Error	t value	p value
(Intercept)	1.576	0.082	19.11	< **0.001**
Muscle & Gills	0.470	0.128	3.665	< **0.001**
Muscle & Liver	0.660	0.130	5.063	< **0.001**
Gills & Liver	0.191	0.141	1.355	0.181

Family = Gamma AIC = 270.3

Table 1. GLM analysis of *A. regius* Fulton's K and total mercury (HgT) concentration in tissues (3 levels within contaminated treatments: liver, muscle and gills) exposed to MeHg contamination (2 levels: non-contaminated and contaminated) for 30 days. Model formula on top, family and respective model AIC in the bottom. Est – Estimates; Std Error – Standard Error. Bold values indicate p < 0.05. For more details please see the R script in Supplemental Data.

	GLM: Liver HgT in function of T * CO_2				***GLM: Muscle HgT in function of T * CO_2***				***GLM: Gills HgT in function of T * CO_2***			
	Est	Std Error	t value	p value	Est	Std Error	t value	p value	Est	Std Error	t value	p value
(Intercept)	2.287	0.151	15.18	< **0.001**	1.520	0.063	24.31	< **0.001**	2.059	0.125	16.74	< **0.001**
T	0.794	0.261	-3.043	**0.010**	0.579	0.088	6.551	< **0.001**	-0.917	0.191	-4.792	< **0.001**
CO_2	-0.295	0.195	-1.514	0.156	-0.201	0.088	-2.268	**0.035**	-0.157	0.162	-0.970	0.350
T * CO_2	1.468	0.326	4.508	< **0.001**	0.627	0.125	5.017	< **0.001**	1.452	0.251	5.799	< **0.001**
Family = Gamma (all)			AIC = 82.0				AIC =59.8				AIC = 73.3	

Table 2. GLM analysis of total mercury concentration (HgT) within each sampled tissue (liver, muscle and gills) of *A. regius* exposed to MeHg for 30 days, under crossed treatments of temperature (T, 2 levels: 19 ºC and 23 ºC) and CO_2 (CO_2, 2 levels: 400 µatm and 1500 µatm). Model formula on top, family and respective model AIC in the bottom. Est – Estimates; Std Error – Standard Error. Bold values indicate p < 0.05. For more details please see the R script in Supplemental Data.

GLM: MDA in function of T * MeHg

	Est	Std Error	t value	*p*
(Intercept)	0.026	0.003	8.055	< **0.001**
T	-0.010	0.005	-2.163	**0.036**
MeHg	-0.004	0.005	-0.954	0.345
T * MeHg	0.014	0.007	2.174	**0.035**

Family = Gaussian AIC = -277.2

Table 3. GLM analysis of malondialdehyde (MDA) build-up in *A. regius* after 30 days exposed to crossed treatments of MeHg contamination (MeHg, 2 levels, non-contaminated and contaminated) and temperature (T, 2 levels: 19 ºC and 23 ºC). Model formula on top, family and respective model AIC in the bottom. Est – Estimates; Std Error – Standard Error. Bold values indicate p < 0.05. For more details please see the R script in Supplemental Data.

GLM: CAT in function of CO_2 * MeHg

	Est	Std Error	t value	*p*
(Intercept)	4.375	0.399	10.96	< **0.001**
CO_2	-0.454	0.564	-0.804	0.426
MeHg	1.482	0.564	2.625	**0.012**
CO_2 * MeHg	1.313	0.818	1.605	0.116

Family = Gaussian AIC = 166.2

GLM: SOD in function of CO_2 * T + CO_2 * MeHg

(Intercept)	9.496	1.040	9.135	< **0.001**
T	3.346	1.200	-2.787	**0.008**
CO_2	-1.264	1.484	-0.852	0.399
MeHg	1.614	1.200	1.344	0.186
T * CO_2	6.319	1.744	3.623	< **0.001**
CO_2 * MeHg	-3.399	1.744	-1.949	0.058

Family = Gaussian AIC = 237.3

GLM: GST in function of T * CO_2 * MeHg

(Intercept)	7.561	0.676	11.19	< **0.001**
T	-1.174	0.955	-1.229	0.227
CO_2	-2.320	0.955	-2.428	**0.020**
MeHg	-2.054	0.955	-2.150	**0.038**
T * CO_2	4.076	1.351	3.017	**0.005**
T * MeHg	2.375	1.351	1.758	0.087
CO2 * MeHg	4.427	1.351	3.277	**0.002**
T * CO_2 * MeHg	-3.422	1.970	-1.737	0.090

Family = Gaussian AIC = 186.1

Table 4. GLM analysis of oxidative stress response (CAT, SOD and GST) in *A. regius* after 30 days exposed to crossed treatments of MeHg exposure (MeHg, 2 levels: non-contaminated and contaminated), temperature (T, 2 levels: 19ºC and 23ºC) and CO_2 (CO_2, 2 levels: 400 µatm and 1500 µatm). Model formula on top, family and respective model AIC in the bottom. Est – Estimates; Std Error – Standard Error. Bold values indicate p < 0.05. For more details please see the R script in Supplemental Data

GLM: Hsp70 in function of Tissues

	Est	Std Error	t value	*p*
(Intercept)	3.605	0.235	15.32	< **0.001**
Gills & Liver	1.607	0.335	4.804	< **0.001**
Gills & Muscle	0.975	0.333	2.929	**0.004**
Muscle & Liver	0.633	0.335	1.890	0.061

Family = Gaussian AIC = 481.5

GLM: Gills Hsp70 in function of T + MeHg

(Intercept)	3.561	0.255	13.99	< **0.001**
T	-0.530	0.299	-1.775	0.083
MeHg	0.622	0.299	2.085	**0.043**

Family = Gaussian AIC = 146.4

GLM: Muscle Hsp70 in function of T * CO_2

(Intercept)	4.671	0.291	16.07	< **0.001**
T	-0.294	0.411	-0.715	0.479
CO_2	-0.955	0.411	-2.323	**0.025**
T * CO_2	-2.331	0.596	3.913	< **0.001**

Family = Gaussian AIC = 137.0

GLM: Liver Hsp70 in function of T + CO_2

(Intercept)	5.376	0.593	9.064	< **0.001**
CO_2	-0.588	0.839	-0.702	0.487
MeHg	1.315	0.839	-1.567	0.125
CO_2 * MeHg	3.627	1.235	2.938	**0.005**

Family = Gaussian AIC = 73.3

Table 5. GLM analysis of heat shock protein 70 (Hsp70) production in *A. regius* tissues (gills, muscle and liver) and, posteriorly within tissues, under crossed treatments of MeHg exposure (MeHg, 2 levels, non-contaminated and contaminated), temperature (T, 2 levels: 19ºC and 23ºC) and CO_2 (CO_2, 2 levels: 400 µatm and 1500 µatm). Model formula on top, family and respective model AIC in the bottom. Est – Estimates; Std Error – Standard Error. Bold values indicate p < 0.05. For more details please see the R script in Supplemental Data.