Anonymous Referee #1

General comments

"This manuscript aims to address a topic of significant importance, namely the interaction between climate change stressors and contamination in coastal regions, and particularly its impact on species of commercial importance. This is certainly a topic of great interest and an area that has been identified as a significant knowledge gap in the field at present. Despite this potential and the undoubted requirement for such a study within the field, regrettably the manuscript presented here does not adequately address this question. As it stands there is insufficient detail presented throughout the methods to adequately appraise what has been done, there appear to be a number of methodological oversights that hamper the interpretation of the results and this has, to a large extent, led to many of the conclusions drawn not being supported by the data. Based on these factors I believe the manuscript at least requires major revisions to include this required detail, as well as restructure the conclusions to match what has actually been undertaken. It would then require re-review to appraise the manuscript in its new form. If it is not possible to include this required detail in full, in its current state the manuscript is not of sufficient quality to be published."

Response: We thank the referee for his suggestions which have served to greatly improve the manuscript. We hope we have now provided sufficient detail on the methodologies employed in this work. We also hope to have clarified some misinterpretations throughout the text. Below, we reply to 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:Abstract, discussion and conclusions – Throughout the manuscript the authors suggest the reduced accumulation of mercury in tissues under combined exposure is due to metabolic depression, and a subsequent reduced apatite/ingestion of food, initiated by elevated CO2. However, the authors do not measure any parameters in the current study that could confirm or counter this suggestion. There is no indication that these fish ingested less food so the conclusions, certainly as they are presented, are unfounded. Reduced accumulation could in fact be caused by a number of different mechanisms in the organism by which elevated COÂn2 augmented Hg accumulation, either by metabolic depression, reduced appetite (could be caused by alternative mechanism), reduced digestive efficiency, reduced uptake across the gut epithelium, greater egestion of Hg or impacts of Hg transport and complexation in plasma to reduce delivery to measured tissues. All are potentially feasible and at present insufficient information is known about this to surmise it is metabolic depression. It is vital to indicate that whilst altered accumulation is noted, which differs between specific tissues, the mechanism is not known. Following this point, the authors have not cited two key

references on ocean acidification and mercury contamination recently published (Li et al Scientific Reports 7;324 2017; and Wang et al ES&T 51:5820 2017). It is possible these were published after initial submission of the current manuscript, but in light of altered mercury accumulation under elevated CO2 these two manuscripts are key as they support the current finding.

Response: The authors acknowledge that no additional parameters were measured to validate the conclusion that lower Hg accumulation under increased CO_2 was due to metabolic depression. Nonetheless, based on previous studies, there are reasons to believe this is the case. A wide range of organisms show metabolic decrease in response to increased extracellular acid–base stress(Kroeker et al., 2010), and especially to simultaneous occurrence of warming and acidification (Harley et al., 2006; Harvey et al., 2013; Rosa et al., 2013; Rosa and Seibel, 2008). Concerning CO_2, theoretically, the prioritization of acid–base regulation and ion regulatory enzyme machinery for CO_2 excretion (e.g. pyruvate kinase) may lead to lower metabolic activity in other enzymes (energy reallocation),as reported in other fish (Perry et al., 1988). As MeHg accumulation rates are positively correlated with metabolic rates (Dijkstra et al., 2013), these results would support the claim that acidification affects toxic compound accumulation rates (Schiedek et al., 2007).

Given our simultaneous exposure to both warming and acidification, which has been shown to undeniably suppress metabolic rates directly (Christensen et al., 2011; Harley et al., 2006; Rosa et al., 2013; Rosa and Seibel, 2008; see also Harvey et al., 2013 and Kroeker et al, 2010) we still hold the conviction that metabolic processes may be at play. However, the authors also acknowledge that they were not aware of the recent research pointed out by the referee, which thoroughly picks apart the causes of behind these mechanisms. We thank the referee for this useful comment and we have altered our interpretation, changing the text:

"However, such effect may be offset by CO_2-linked decreases in mercury accumulation (Sampaio et al., 2016; Schiedek et al., 2007; Wang et al., 2017)" (Page 2, Lines 29-31)

"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)

"In general, warming conditions enhanced MeHg accumulation but CO_2-linked impacts countered this effect." (Page 12, Lines 28-29)

References

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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.

Schiedek, D., Sundelin, B., Readman, J. W. and Macdonald, R. W.: Interactions between climate change and contaminants, Mar. Pollut. Bull., 54, 1845–1856, doi:10.1016/j.marpolbul.2007.09.020, 2007.

Wang, M., Lee, J.-S. and Li, Y.: Global Proteome Profiling of a Marine Copepod and the Mitigating Effect of Ocean Acidification on Mercury Toxicity after Multigenerational Exposure, Environ. Sci. Technol., 51, 5820–5831, doi:10.1021/acs.est.7b01832, 2017a.

Comment #2: Abstract, discussion and conclusions – Similarly to the point above, the authors repeatedly suggest elevated H+ impacts mercury accumulation/toxicity at a molecular level, but no acid base measures

were made. Also it is a common misunderstanding that elevated CO2 results in chronic acidosis in fish plasma, this is not the case. Elevated CO2 results in acute acidosis which is rapidly compensated for by an elevation in bicarbonate, returning the plasma H+ to normal levels. Therefore the suggestion that elevated H+ impacts on mercury toxicity/accumulation is not supported, especially as acid-base parameters are not presented that counter this common response noted in acid-base compensating species such as fish. The authors need to again re-interpret data and re-write conclusions to better reflect the demonstrated results and not make broad unsupported conclusions, pinned loosely on previously published literature that has been misinterpreted/misunderstood.

Response: We would like to point out that we never said that acidosis was present in a long-term perspective, nor did we assume that fish are not able to acid-base compensate, a mechanism that is already extensively described (Brauner and Baker, 2009; Heuer and Grosell, 2014; Michaelidis et al., 2007; among many more). In fact, besides some logistical and time constrains, that was the main reason why no acid-base measurements were performed. Having said that, acid-base compensation occurs mainly by increasing bicarbonate (HCO_3^-) levels in both blood and cellular, which in turn leads to a normalization of intracellular and extracellular pH (Heuer and Grosell, 2014; Michaelidis et al., 2007). The chemical equation that underpins this reaction is as follows:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

Thus, despite pH being normalized by balancing the ratio between $H^+ + HCO_3^-$ and H_2CO_3 (it generally stabilizes at ~0.05/0.1 units lower than in normocapic conditions), it is important to note that H^+ levels in the organism are still increased relatively to basal levels, especially in long-term acclimations to hypercapnia-**where there is a constant influx of H^+ ions** (Heuer and Grosell, 2014; Michaelidis et al., 2007). Moreover, due to cell prioritization, intracellular and extracellular pH often display significantly different values: the former is up-regulated to normocapnic levels or higher, while the latter generally stabilizes at lower pH (ΔpHcan reach~0.3-0.7) (Brauner and Baker, 2009; Heuer and Grosell, 2014). This also partially contributes to increased H^+ levels. As our reasoning is grounded on molecular interactions (both oxidative stress-inducing and ROS-mitigating) of increased H^+ chemical reactions (see also Dean, 2010), it does not imply for fish acid-base compensation to fail.

In light of the new recent studies mentioned by the referee, we have introduced some new considerations to our Abstract/Discussion/Conclusion. The reason we do not believe that the lysozyme-autophagy pathway (Wang et al., 2017) is solely responsible for the antagonistic relationship between stressors is that it does not account for hypercapnia-induced oxidative stress and chaperone activation. Within this context, we have rephrased our interpretations and changed the text accordingly:

In the Abstract:

"Together with CO_2-promoted removal of damaged proteins and enzymes, we argue that simultaneous increase in hydrogen (H^+) and reactive oxygen species (e.g. O_2^-) radicals is partially compensated through chemical reaction equilibrium balancing." (Page 1, Lines 26-29)

In the Discussion:

"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 mechanism may contribute to alleviate not only Hg induced stress, but also warming-related oxidative stress. We also argue that this antagonistic relation can be partially explained by 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 (Heuer and Grosell, 2014; 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. However the production of O_2^- and further complementary ROS 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." (Page 11/12, Lines 31/1-10)

"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. Given that Hsp70 production can also be stimulated by high ionic (e.g. H^+) concentrations (Feder and Hofmann, 1999), we reason that the same additional mechanism by which hypercapnia potentially modulates oxidative stress can be applied for heat shock response" (Page 12, Lines 18-22)

In the Conclusions:

"In fact, despite negative effects prompted as a sole stressor, acidification consistently elicited antagonistic responses to temperature and contamination effects on oxidative stress (including heat shock response), which may be explained by stimulated removal of damaged proteins and organelles (Wang et al., 2017). Moreover, we also argue that the mechanistic interactions found are coadjuvanted by the coinciding increase of 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. $H^+ + OH^- \rightleftharpoons H_2O$)." (Page 12/13, Lines 29/1-6)

References

Brauner, C. J. and Baker, D. W.: Patterns of Acid–Base Regulation During Exposure to Hypercarbia in Fishes, in Cardio-Respiratory Control in Vertebrates, edited by M. L. Glass and S. C. Wood, pp. 1–546, Springer Berlin Heidelberg., 2009.

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Michaelidis, B., Spring, A. and Pörtner, H. O.: Effects of long-term acclimation to environmental hypercapnia on extracellular acid-base status and metabolic capacity in Mediterranean fish Sparus aurata, Mar. Biol., 150(6), 1417–1429, doi:10.1007/s00227-006-0436-8, 2007.

Comment #3: Discussion – The authors suggest the Fulton condition may diminish under mercury contamination. Whilst AIC indicates the best fit model as slightly negative the statistic (p-Value) clearly indicates no significant effect and therefore suggesting this is not the case may mislead readers to interpret a result that is not supported statistically, even if it may support a previous publication.

Response: Following the reviewer's instructions, we have removed any mentioning of negative effects on the Fulton condition. We have changed the introductory paragraph of the Discussion to:

"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. 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." (Page 10, Lines 14-19)

Comment #4: Intro and methods - The justification of mercury, and methylmercury, in fish from coastal regions is insufficient. There is no quantification of levels within the environment from different regions globally, how coastal compares to open ocean and how this then translates into a burden for fish populations. As it stands this is not adequate for a contaminant manuscript, and belies the statement that an environmentally relevant concentration was used, as stated in the methods. What is an environmentally relevant concentration, where does the level chosen fit with measured environmental levels from different regions globally, and even just within the region the study was undertaken in. Finally, if the route of uptake is solely dietary for fish then how do environmental levels correspond to burdens in prey species and thus exposure in the experimental organism? Is the level chosen a typical contaminant level in prey species in an impacted environment or the level in water/sediment? This needs clarifying, and fully justifying in relation to existing literature and levels previously used.

Response: Mercury (originating mainly from industrial residue) accumulates in the sediments of river basins and estuaries (Mason, 2001). Posteriorly, it is transported to the open ocean via particulate and dissolved sediments in water currents and accumulated within animals, but in much less quantity (Guentzel et al., 1996). Thus, it is logical that fish which often make use of estuaries are more vulnerable to mercury accumulation, as we have stated in the Introduction of the manuscript (Page 2, Lines 18-20 and Page 3, Lines 21-22). As the referee correctly inferred, the concentrations of mercury used 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 of the meagre(e.g. Cardoso et al., 2014; Nunes et al., 2008). These mercury concentrations can also be found in other areas globally, e.g. Florida, USA (Kannan et al., 1998).

We have changed the text in order to provide a more comprehensive picture:

"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 mimic 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.

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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.

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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 #5: Methods - Following this, the total amount of mercury (mg per kg of food), is higher than the content of methylmercury added as an additive (8.02 MeHg, 8.28 HgT). This is not possible. Also how were these levels measured (or is it nominal)?

Response: We assure the reviewer that this is standard for all scientific works where, being the most bioaccumulated form of mercury in the environment, methylmercury (MeHg) is used (Maulvault et al., 2016, 2017; Sampaio et al., 2016; Wang et al., 2013, 2017b). On top of naturally occurring demethylation, higher total mercury concentration is due to the ubiquity of mercury, under several (organic and inorganic) forms, in the natural environment. A standard feed diet is composed of fish meals, oils and other compounds, which already contain a certain quantity of mercury (not only methylmercury, but also in its other chemical forms). Naturally, a control feed, where no spiking is performed, contains trace levels of mercury. Thus, when spiking a diet with MeHg, adding these facts, it is common for the total amount of mercury to be higher than that of methylmercury (e.g. see studies referenced).

Lastly, the procedure for the measurement of MeHg is similar to HgT, explicit in section "2.2 Total mercury and Methylmercury accumulation" (Page 6, Lines 5-18).

For the sake of clarity, we rephrased:

"Afterwards, HgT (all samples) and MeHg (feed samples) were determined (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} ww." (Page 6, Line 5-8)

And added:

"Feed composition, manufacturing and MeHg spiking processes were executed as described by Maulvault et al. (2016). Fish were fed two to three times a day and total feed 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 feed quantity also minimized food remains, which, in case of existing, were siphoned together with fish faeces after feeding." (Page 5, Line 5-12)

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.

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Wang, X., Wu, F. and Wang, W.-X.: In Vivo Mercury Demethylation in a Marine Fish (*Acanthopagrus schlegeli*), Environ. Sci. Technol., (May), acs.est.7b00923, doi:10.1021/acs.est.7b00923, 2017b.

Comment #6: *Methods (page 4 lines 1-7) - The description of the conditions, and particularly their maintenance is not sufficient. It states ammonia, nitrate and nitrite were regularly monitored and kept within recommended levels. How was this tested, what were the accepted levels and what were the levels measured within the experiment? Also how were high levels mitigated against and how often? Furthermore, it mentions salinity was kept at 35.0 ± 1.0 g/l NaCl? The probe listed is a conductivity probe so does not measure in g/l of NaCl but gives a conductivity measure or salinity as a psu. Also how was salinity maintained? i.e. is this addition of deionised water to compensate for evaporation? Or addition of additional NaCl? The description is confusing, and could be interpreted as additional NaCL addition. Any further addition of NaCl would significantly alter osmolality thus this needs clarifying to explain if input water fluctuated in salinity. A better description of this process is therefore required.*

Response: We apologize for not having provided more detail on these matters, but we have been said to be overzealous with these descriptions in recent publications. However, it is our pleasure to fill the gaps the referee points out in this section.

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}).

Salinity was not measured through a conductivity probe, we apologize for the omission. We opted instead for a refractometer (V2, TMC Iberia, Portugal) and took daily measurements as with temperature and pH. Salinity was also incorporated in the calculation of seawater carbonate chemistry (Table S1). We acknowledge the mistake (g/l) and have removed salinity units (see below).

The addition of deionised water or any kind of water except sea water would modify carbonate chemistry and render our pH manipulation useless (Cornwall and Hurd, 2015). All potential fluctuations in both these parameters were solved by the seawater flux, and in the case of nutrients, 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 parameters such as salinity and nutrients. Thus, the mitigation of

potential problems was done *a priori* and no additional action was needed during the course of the experiment.

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). Temperature and pH (multiparametric probe, Multi3420 SET G, WTW) were measured daily, directly in the holding tanks. Photoperiod was fixed at 12 h light : 12 h dark." (Page 4, Lines 5-14).

References

Cornwall, C. E. and Hurd, C. L.: Experimental design in ocean acidification research: problems and solutions, ICES J. Mar. Sci., 73, 572–581, 2015.

Comment #7: Methods - There is no measure (or data presented) of methylmercury or total mercury in experimental water. This is a major omission, and gives no indication as to what proportion of the contaminant leaches from food into water, particularly if any food remains uneaten and in the tank for any time. It also prevents the discussion of amounts of methylmercury that egested immediately into the water by this fish, not being taken up or bioaccumulated.

Response: Our previous study showed that, contrary to inorganic mercury, the quantity of methylmercury leeched from the feed to the water was **below detection levels,** making water measurements **irrelevant** (Maulvault et al., 2016). In other words, although measurements in the water are important when working with inorganic mercury, methylmercury is a more strongly lipophilic and hydrophobic molecule. It preferentially adheres to sediment and accumulates in the tissues of animals (i.e. fish) via prey (Mason, 2001). Moreover, the quantity of food administered (1 % fish weight per fish) is standard for aquaculture and has been calculated so that remains are minimum. In the rare occasions food was not ingested, **it was immediately siphoned together with fish faeces**.

We added this information in the text:

"Feed composition, manufacturing and MeHg spiking processes were executed as described by Maulvault et al. (2016). Fish were fed two to three times a day and total feed 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 feed quantity also minimized food remains, which, in case of existing, were siphoned together with fish faeces after feeding." (Page 5, Line 5-12)

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 (Dicentrarchuslabrax) in a warmer environment, Environ. Res., 149, 77–85, doi:10.1016/j.envres.2016.04.035, 2016.

Mason, R. P.: The Bioaccumulation of Mercury, Methylmercury and Other Toxic Elements into Pelagic and Benthic Organisms, in Coastal and Estuarine Risk Assessment, edited by N. Newman, M. Roberts jr, and R. Hale, pp. 127–149, Lewis Publishers, Boca Raton, Fl, USA., 2001.

Comment #8: *Throughout - Given the commercial importance of the species, one surprising oversight is the fact that no discussion on different tissue burdens were made with respect to human consumption and climate change impacts. The only place this is alluded to is in the title! This is particularly relevant given the possibility that elevated CO2 reduces Hg accumulation possibly reducing transfer of hg into humans directly via consumption of muscle tissue, which could be an important result. This would provide some wider context in which to place the importance of this study generally, as well as contaminant/climate changes studies more generally.*

Response: We thank the referee for this thoughtful comment and have introduced considerations on this matter:

"From a consumer perspective, our study showed that the counter-acting CO_2 effect (hampering warming-stimulated Hg accumulation) was consistent in the muscle, the main tissue ingested by human population. Since this is the most relevant tissue for commercialization, such results constitute an important finding in the area of seafood safety, worthy of further research." (Page 11, Line 16-20)

"Further knowledge on climate change and contamination impacts on fish ecophysiology (and biochemical stress-coping mechanisms) will help towards better comprehension of future fish stocks' health condition and tissue-dependent contaminant accumulation, 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." (Page 13, Line 8-15)

Technical corrections:

Technical correction #1: *Page 1, Line 18-19 – Sentence beginning "Despite the more than likely co-occurrence..." is weak and doesn't read well. Needs stronger justification (see above)to enable stronger conviction in abstract, as well as explicitly highlight that contaminant/climate change stressor interactions are largely overlooked, rather than just "these stressors".*

Response: We rephrased:

"Future interactive effects between 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." (Page 1, Lines 16-19)

Technical correction #2: *Page 1, Line 29 – should read mechanisms not mechanism*

Response: Changed.

Technical correction #3: *Page 2, Line 2 (and throughout) – should be CO2 sub-scripted, this error occurs in a number of positions throughout manuscript, also sometimes is sub-scripted so inconsistent.*

Response: Corrected.

Technical correction #4: *Page 2, Line 4-5 –I would argue greenhouse gas effect is increasing global temperatures, and this is resulting in projected further increase (already increased by 0.76 ºC from pre-industrial) in surface ocean temperature of . . . by end of the century.*

Response: Changed to:

"Moreover, conjointly with other "greenhouse" gases, increased CO_2 has triggered a continuous rise in mean ocean temperatures (nowadays increased by 0.76ºC from pre-industrial values), and predictions point to a further 0.3-4.8 °C increase by the end of the century (IPCC, 2014)." (Page 2, Lines 2-6)

Technical correction #5: *Page 2, Line 22 – Should read ". . .Sampaio et al., 2016) and ultimately mortality (Coccini et al., 2000)."*

Response: Changed.

Technical correction #6: *Page 3, Line 7 – protein not proteins*

Response: Changed.

Technical correction #7: *Page 3, line 11 – responses not response*

Response: Changed.

Technical correction #8: *Page 3, Line 16 – remove the before estuaries*

Response: Removed.

Technical correction #9: *Page 4, Line 10 – should be pH controllers not controller*

Response: In this case, although multiple pH probes were used, all were connected to a single pH controller, i.e. a Profilux system (± 0.1, Profilux 3.1N, GHL). However, we have changed phrasing for the sake of clarity:

"We used a Profilux system (± 0.1, Profilux 3.1N, GHL) as pH controller, connected to each tank by individual pH probes." (Page 4, Lines 17-18)

Technical correction #10: *Page 5, Line 5 – Length3 should be super-scripted*

Response: Changed.

Technical correction #11: *Page 5, Line 19 – remove with before nitric acid*

Response: Removed.

Technical correction #12: *Page 5, Line 23 – should be gill not gills*

Response: Changed.

Technical correction #13: *Page 5, Line 25 – remove posteriorly*

Response: Removed.

Technical correction #14: *Page 5, Line 26 – rewrite as "..response concentrations, quantified" removing were*

Response: Changed.

Technical correction #15: *Page 6, Line 18 – assume is potassium periodate not potassium per iodate*

Response: Corrected.

Technical correction #16: *Page 6, Line 23 (and page 7, line 16) – mg-2 needs super-scripting*

Response: Done.

Technical correction #17: *Page 6, Line 25 – insert space before Superoxide*

Response: Done.

Technical correction #18: *Page 7, Line 5 – is the % inhibition of SOD activity calculated as maximum inhibition, average inhibition at each 5 minute time point or from initial and final, just measured every 5 minutes over 25 minutes so potentially have different rates of inhibition and total overall inhibition over this time course*

Response: It is the average inhibition from initial to final (25 minutes, 5 minute readings are used to create the slope).We included the information:

"…, which allowed the assessment of inhibition percentage per minute (averaged from 25 minutes),…" (Page 7, Line 28-29)

Technical correction #19: *Page 7, Line 23 – insert space before and*

Response: Done.

Technical correction #20: *Page 8, Line 2 – insert space in mg-1total*

Response: Done.

Technical correction #21: *Page 9, Line 19 (and other places) – A. regius needs italicising*

Response: Corrected throughout the manuscript.

Technical correction #22: *Page 10, Line 2 – notoriously is an odd choice of words, suggest just removing as reads fine without replacing*

Response: Changed according to referee's suggestions.

Technical correction #23: *Page 10, Line 23 (and page 11, line 15) – H20 needs subscripting*

Response: Done.

Technical correction #24: *Page 18 – Why is the x-axis reversed on figure 1, d, compared to b and c. This confuses comparisons.*

Response: Indeed, we apologize for the mistake and have corrected it. See new Figure 1 in the marked manuscript.

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

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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. ~~FDespite 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 ($\Delta T = 4$ ºC) and acidification ($\Delta pCO_2 = 1100$ 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 ($\simeq$400 CO_2 µatm nowadays), and are expected to reach approximately 1000 CO_2 µatm by the year 2100 (IPCC, 2014). Moreover, ~~Increased CO₂,along~~conjointly with other "greenhouse" gase~~ss~~, increased CO_2 has trigger~~eds~~ 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~~ decrease~~s,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.

5　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

10　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

15　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 response~~s~~ (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

20　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,

25　here we performed a 30-day acclimation experiment to investigate organ-dependent Hg accumulation (gills, liver and muscle) under a warming ($\Delta T = 4$ ºC) and acidification ($\Delta CO_2 = 1100$ 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~~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 ~~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). ~~g l⁻¹ and the photoperiod 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, CH₃ClHg, 99.8 %, Sigma-Aldrich, solubilized previously in ethanol). Given our dietary option, ecologically relevant MeHg concentrations ~~was~~ere 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 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). ~~Given our dietary option, MeHg concentration was chosen based on levels found in 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 ℃, $CO_2 \simeq$ 400 µatm), fish were kept during 30 days under crossed-treatments of ocean warming ($\Delta T = 4$ °C), acidification ($\Delta pH = 0.5$ units, i.e. $\Delta pCO_2 = 1100$ 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 x (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

2.3.1 Preparation 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 mM KCl, 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).

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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 ℃. 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,

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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⁻¹total 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.

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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~~icalcondition~~ 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,~~ taking ~~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

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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.

8 Competing 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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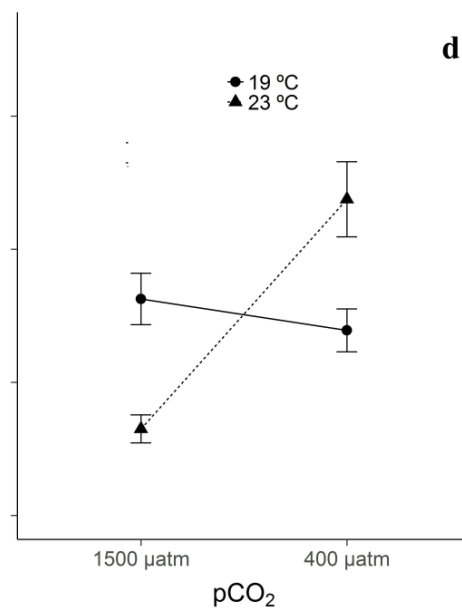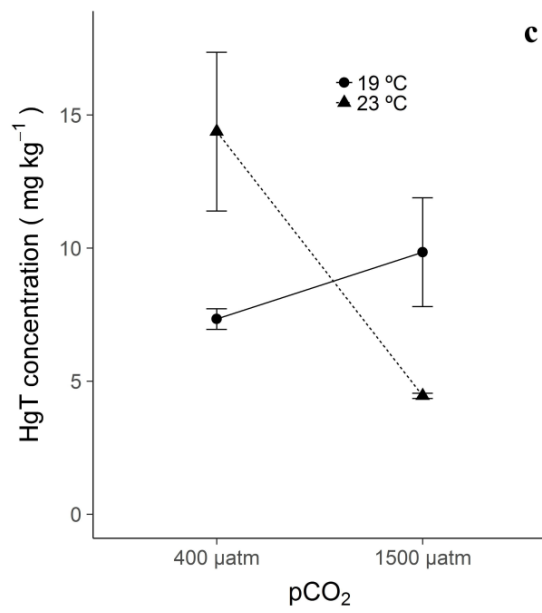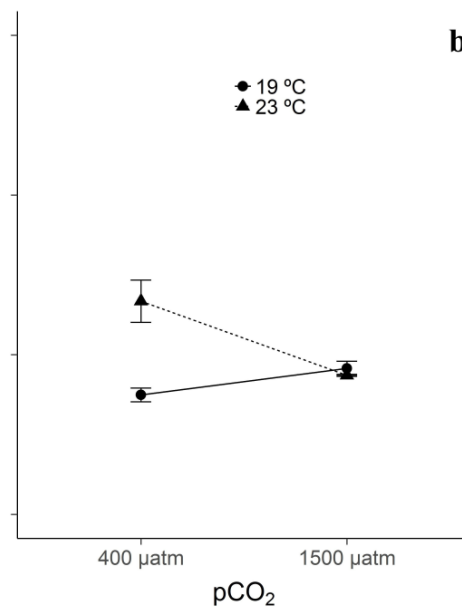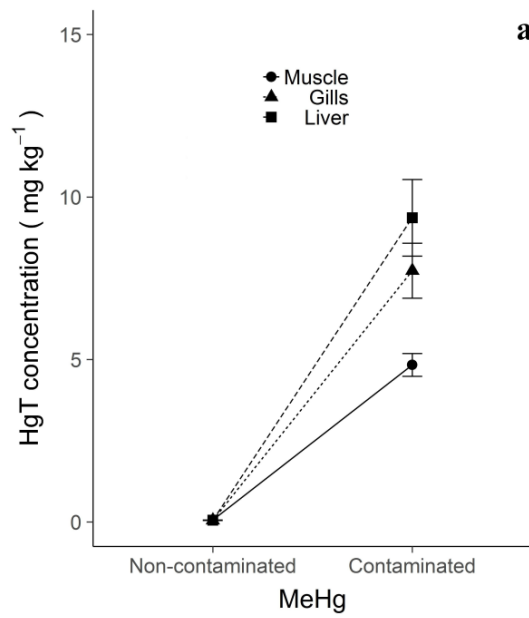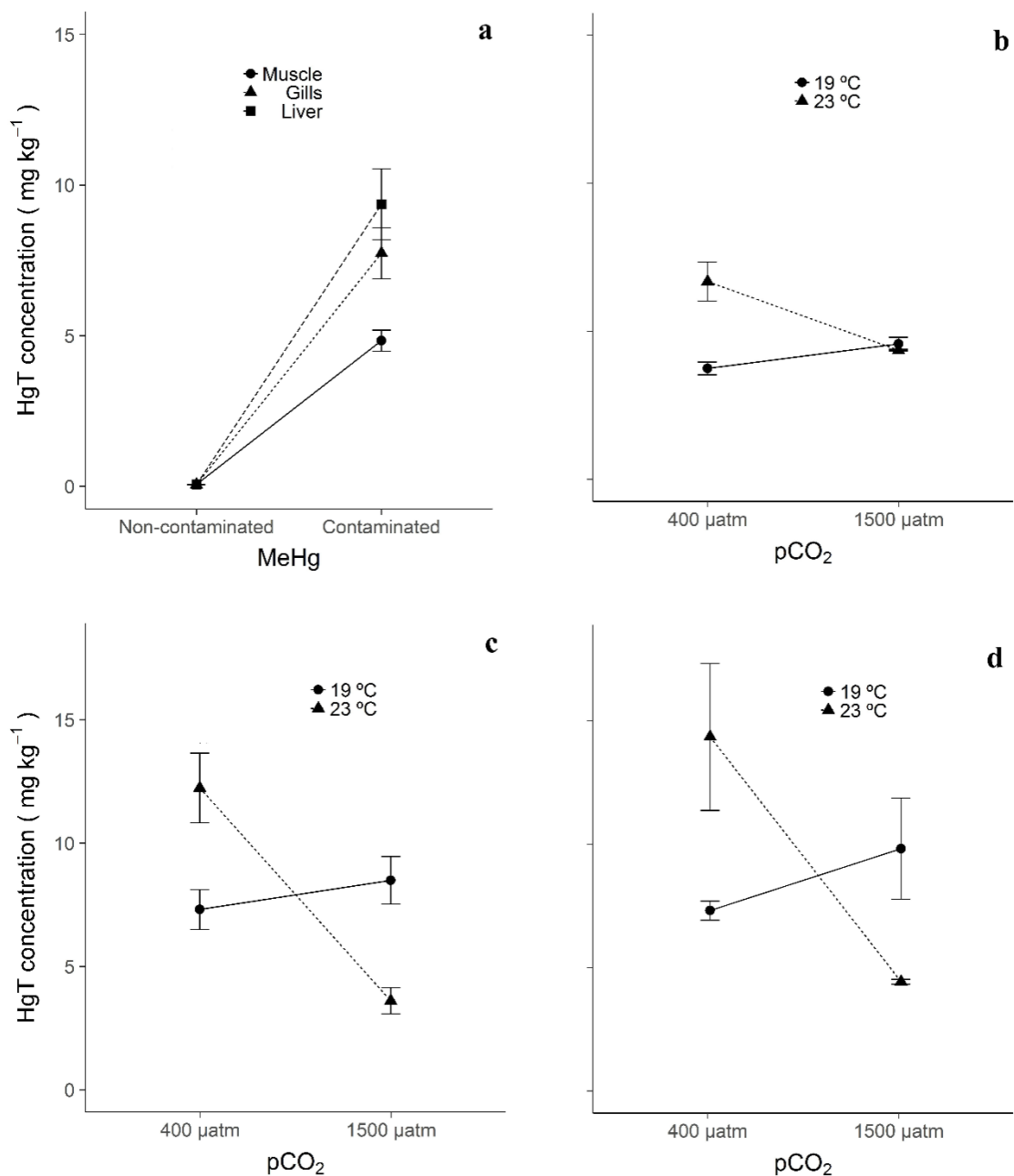
Figure 1. Total mercury (HgT) accumulation (mean ± SE) in *A. regius*: a) Differences among tissues (muscle, gills and liver); and shaped by interactions between temperature (19 and 23 ºC) and CO_2 (400 and 1500 µatm) within b) muscle, c) gills and d) liver, respectively. Graphs were plotted according to significant factors yielded by GLM analysis described in Table 1 and 2, respectively.
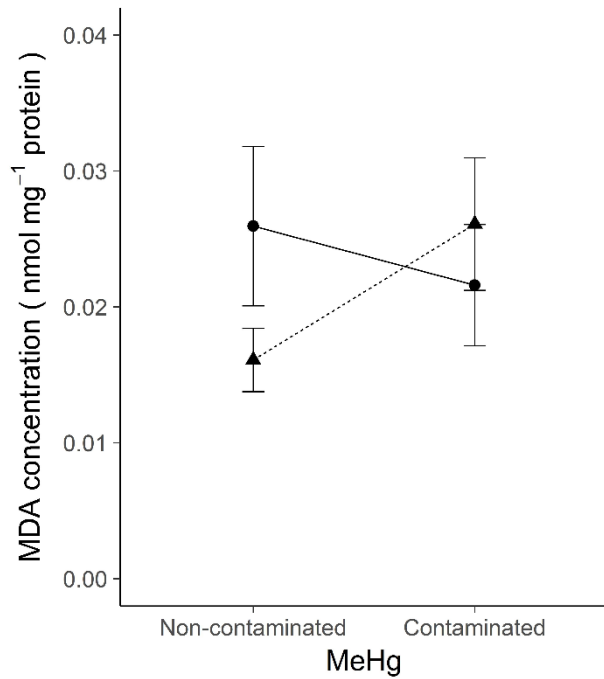
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Figure 2. Malondialdehyde (MDA) build-up concentrations (mean ± SE) in *A. regius* <u>muscle</u> driven by an interaction between MeHg contamination (Non-contaminated and contaminated) and temperature (19 and 23 ºC). Graphs were plotted according to significant factors yielded by GLM analysis described in Table 3 and 4, respectively.
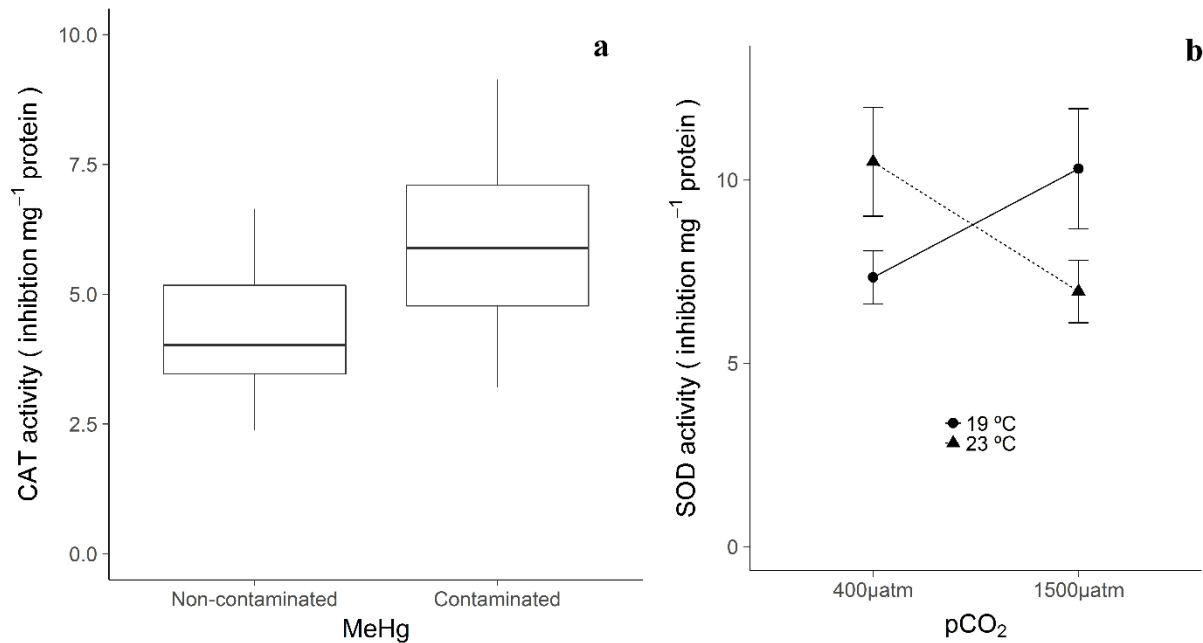
Figure 3. a) Catalase (CAT) enzyme activities (mean ± SE) driven by MeHg contamination (Non-contaminated and Contaminated). b) Superoxide dismutase (SOD) activities (mean ± SE) in *A. regius* <u>muscle</u> driven by an interaction temperature (19 and 23 ºC) and CO_2 (400 and 1500 µatm). Graphs were plotted according to significant factors yielded by GLM analysis described in Table 4.
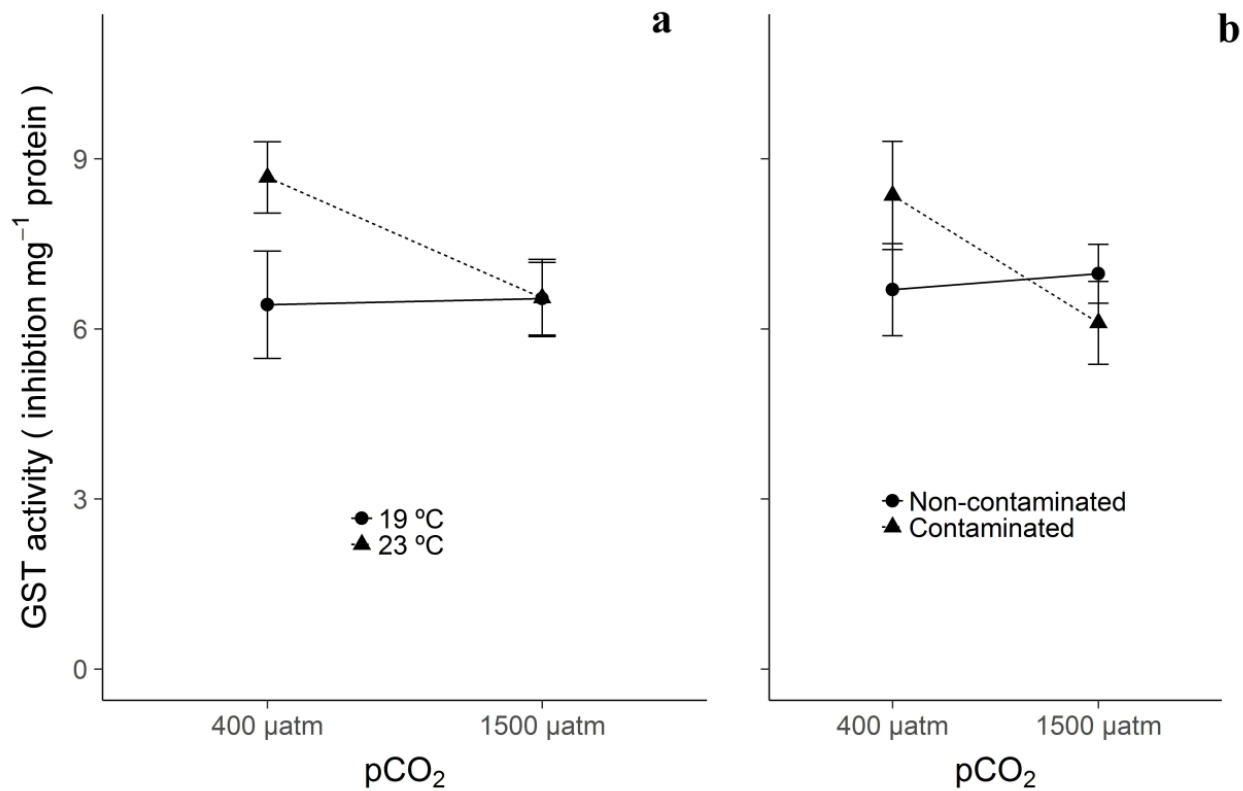
Figure 4. Glutathione S-Transferase (GST) activities (mean ± SE) in *A. regius* muscle driven by: a) an interaction between temperature (19 and 23 ºC) and CO_2 (400 and 1500 µatm); and b) an interaction between MeHg contamination (Non-contaminated and contaminated) and CO_2 (400 and 1500 µatm). Graphs were plotted according to significant factors yielded by GLM analysis (triple interaction) described in Table 4.
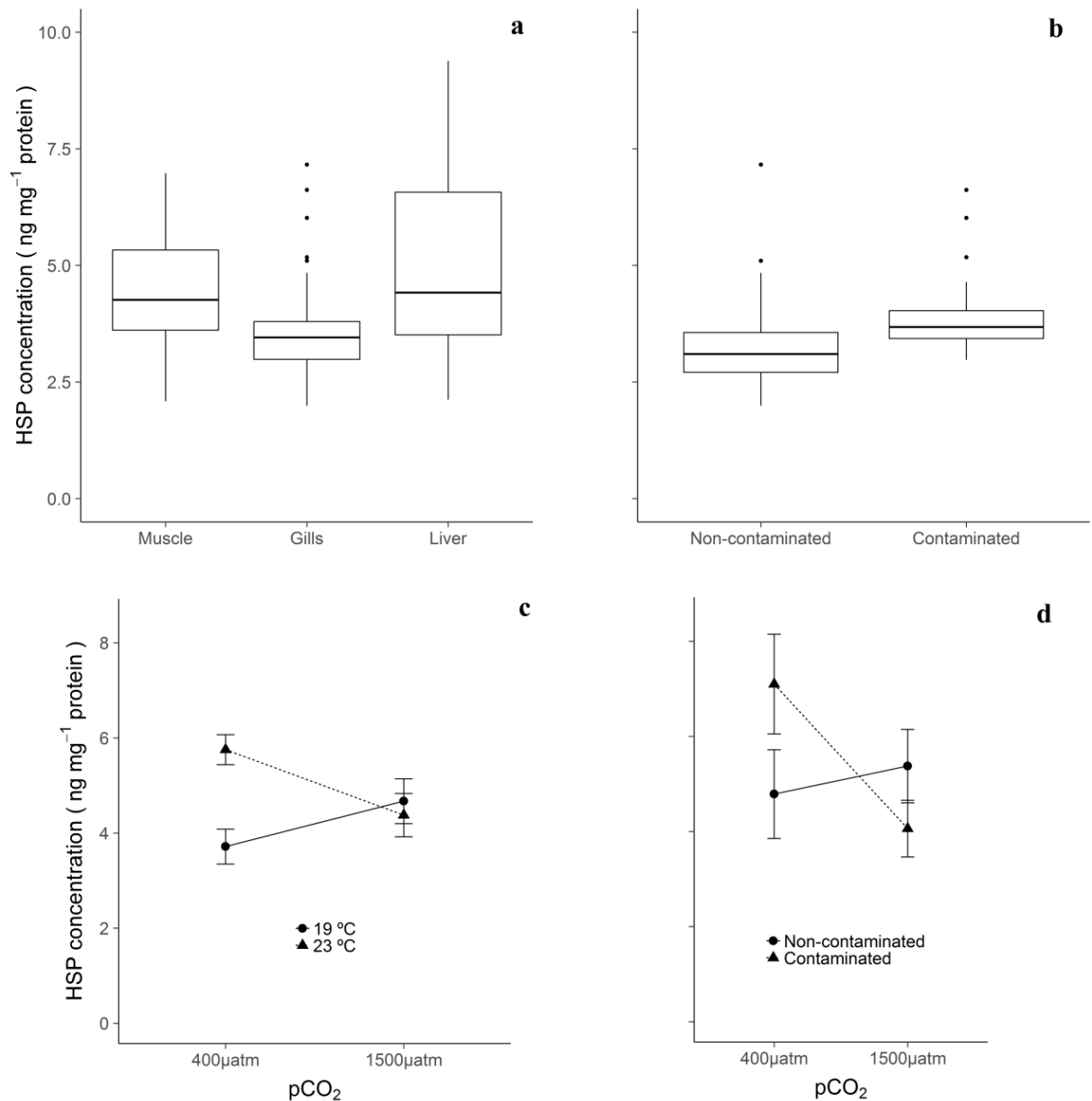
Figure 5. Heat shock protein70 (Hsp70) concentrations (mean ± SE) in *A. regius*: a) tissues; b) in the gills shaped by MeHg contamination (Non-contaminated and Contaminated) and CO_2 (400 and 1500 µatm); in the c) muscle shaped by an interaction between temperature (19 and 23 ºC) and CO_2 (400 and 1500 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.

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	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.

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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

	Est	Std Error	t value	p
(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

	Est	Std Error	t value	p
(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.