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Reviewer #1

- 1- Title, the expression "elevated temperatures" is not a clear expression, perhaps authors could search for another expression.

The expression "elevated temperatures" has been replaced by "temperature"

- 2- Page 7, Lines 145-...., results on metal contents must be adjusted attending at significant figures: 0.378 ± 0.05 must be expressed as 0.38 ± 0.05 11.63 ± 0.74 must be expressed as 11.6 ± 0.7 172.64 ± 8.89 must be expressed as 173 ± 9 40.14 ± 2.78 must be expressed as 40 ± 3 0.052 ± 0.01 must be expressed as 0.05 ± 0.01 0.15 ± 0.09 it is OK 138.94 ± 8.76 must be expressed as 139 ± 9

Values have been expressed as suggested by reviewer.

- 3- Table 3. Secondary horizontal lines could be eliminated.

Secondary horizontal lines have been eliminated.

Reviewer #2

- 4- Although well organized, the manuscript should be reviewed from a linguistic point of view, being misspelled many words and not clear some sentences.

Manuscript has been reviewed from a linguistic point of view. Changes made are marked in the revised manuscript.

- 5- The procedure used for the determination of the metallothionein content in the liver must be described.

Procedure for metallothionein determination has been described.

- 6- Possibly, metallothionein content and induction should be investigated also in the muscle.

Although we agree with reviewer #2 in the interest of determining metallothionein induction also in muscle, liver is recognized to have a greater importance in metal handling than muscle. Furthermore, there are not muscle samples left from this experiment.

**Long-term effect of ~~elevated~~ temperature on bioaccumulation of dietary
metals and metallothionein induction in *Sparus aurata*.**

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Abstract

Previous studies have demonstrated that the commercial feed of aquacultured
fish contains trace amounts of toxic and essential metals which can accumulate
in tissues and finally be ingested by consumers. Recently rising temperatures,
associated to the global warming phenomenon, have been reported as a factor
to be taken into consideration in ecotoxicology, since temperature-~~de~~-dependent
alterations ~~on~~in bioavailability, toxicokinetics and biotransformation rates can be
expected. *Sparus aurata* were kept at 22°C, 27°C and 30°C for three months in
order to determine the temperature effect on metallothionein induction and
metal bioaccumulation from a non-experimentally contaminated commercial
feed. A significant temperature-dependent accumulation of cadmium (Cd),
copper (Cu), mercury (Hg), zinc (Zn), lead (Pb) and iron (Fe) was found in liver,
together with that of manganese (Mn), Fe and Zn in muscle. Hg presented the

25 highest bioaccumulation factor, and essential metal homeostasis was disturbed
26 in both tissues at warm temperatures. An enhancement of hepatic
27 metallothionein induction was found in fish exposed to the highest temperature.

28 **Keywords:** metal; metallothionein; bioaccumulation factor; temperature; *Sparus*
29 *aurata*

1. Introduction

The aquatic ecosystems are undergoing a warming in their deep and surface waters, a fact which may have significant consequences on the organisms inhabiting them (Noyes et al., 2009). Gilthead sea bream *Sparus aurata* is a cosmopolitan species distributed ~~in~~throughout the Mediterranean and ~~in the of~~ the NE Atlantic, ~~and~~ which is of great economic interest since it is one of the ~~main~~principal species of Mediterranean aquaculture. ~~As~~Being an ectothermic species, it is vulnerable to the effects of temperature variations on its metabolism and physiology. These variations may produce changes in the toxicokinetics, bioavailability, biotransformation, homeostasis, absorption rate and elimination of different compounds (Douben, 1989; Köck et al., 1996; Yang and Chen, 1996). Other key physiological mechanisms, such as respiration, feeding rate, growth and reproduction may also be affected (Bowen et al., 2006; Heugens et al., 2001). The thermic induced variations in the toxicokinetics of pollutants, together with the increase ~~of~~in exposure to the same as a consequence of ~~climate~~ warming ~~climate~~ (Carrie et al., 2010) may present a risk for the development and survival of species of commercial interest, affecting the quality of the end product (Noyes et al., 2009).

In cultured fish, it has been demonstrated that the ~~feed is the~~ principal source of contamination by metals ~~is the feed~~. Cadmium (Cd), lead (Pb) and mercury (Hg), among others, are potentially toxic and tend to accumulate in the tissues, which in the end are ingested by the consumers (Fernandes et al., 2009; Maule et al., 2007; Creti et al., 2010). Their accumulation in the organisms depends on the concentration, route of absorption, environmental conditions and other

intrinsic factors (Bowen et al., 2006; Chowdhury and Blust, 2001; Jezierska and Witeska, 2006; Karakoç and Dinçer, 2003; Lemus and Chung, 1999).

Due to the growth in the activity of aquaculture ~~activity~~ in recent decades, it has become of special relevance to learn the influence of the increase in temperature on the possible routes of absorption, accumulation and elimination of metals in these organisms in the context of global warming. ~~The temperature~~ Temperature may be a determining factor in the capture, transporting and metabolism of the metals incorporated through the feed, both of the essential metals which may become toxic at high concentrations in the tissues, and of the non-essential metals.

Metallothionein (MT) is a low-molecular-weight metal binding protein and is known to play an important role in protection against heavy metal toxicity. In addition to the detoxification of toxic metals such as Cd and Hg, MT is involved in the maintenance of homeostasis of essential trace elements such as zinc (Zn) and copper (Cu) (Coyle et al., 2002; Hamilton and Mehrle, 1986). Its role in the protection against xenobiotics or in the cellular protection against oxidative stress should be underlined (Coyle et al., 2002; Van Cleef-Toedt et al., 2001).

Although its synthesis is related to the metal exposure, its levels can be affected by endogenous and exogenous factors such as the reproductive cycle or the temperature (Van Cleef-Toedt et al., 2001). Variations in the water temperature could ~~modify~~, directly or indirectly, modify the behavior of this protein as regards the bioaccumulation of metals, as well as its participation in toxicokinetic processes (Baykan et al., 2007; Gorbi et al., 2005; Rotchell et al., 2001).

Given the lack of information as regards the effect of temperature on the bioaccumulation of metals via dietary sources, and on the synthesis of MT, an

understanding of these processes in the light of the problem of global warming and its repercussions on species of commercial interest such as *Sparus aurata* is required. As such, the aims of this work are:

1) To determine the effect of temperature on the bioaccumulation of essential (Cu, Fe, Mn and Zn) and non-essential (Cd, Hg and Pb) metals experimentally via a non-contaminated commercial feed.

2) To discover whether ~~the~~ temperature has any influence on the homeostasis of the essential metals.

3) To elucidate the role of metallothionein ~~role~~ in the above mentioned temperature induced changes.

2. Materials and Methods

2.1. Animal collection and maintenance

Adult *Sparus aurata* were distributed and acclimated in 500 L tanks containing seawater (~~37‰~~, ~~‰~~) at a constant temperature of 22°C, with continuous aeration and natural photoperiod, in a closed circuit for two months ~~before starting~~prior to the experiment. Subsequently, the temperature of two of the experimental groups was gradually increased to reach 27°C and 30°C respectively. Control groups of fish were maintained at 22 °C throughout the experiments. The animals were fed with commercial pellets (1.5% of body mass per day) and the survival percentage was 99%. Fish were kept under constant conditions for three months. After this period, 6-7 fish were removed at random from each experimental group and ~~held~~placed in water containing 30 mg L⁻¹ of anesthetic clove oil. Lengths and weights of gilthead sea bream were recorded. Fish were sacrificed; livers and a piece of dorsal muscle tissue were dissected and immediately frozen in liquid nitrogen and stored at -80 °C.

2.2. MT determination by differential pulse polarography

Approximately 0.2 g wet weight portions of frozen liver were homogenized using ultra-turrax in 20 mM Tris-HCl buffer, 1 mM DTT and 0.2 mM PMSF pH 8.6 in an ice bath. The homogenates were centrifuged at 30 000 g for 45 min at 4°C. The supernatant was heated at 80°C for 10 min in order to denature high molecular weight proteins and subsequently centrifuged at 30 000 g for 45 min at 4°C. The heat-treated supernatant, containing thermally stable MT, was separated from precipitated proteins. MT was measured using differential pulse polarography ~~as described by~~ (Ureña et al., 2007).

An aliquot of the heat-treated supernatant was added to the polarographic cell, containing 20 mL hexammincobalt chloride buffer (the supporting electrolyte), together with Triton-X (0.025% v/v). The cell was purged for 2 min with purified N₂ prior to analysis. The polarographic response was measured during a potential scan between -1.38 V and -1.7 V (Model 757 VA Computrace Analyser, Methrom, Switzerland) in SMDE mode. Quantification of MT was performed by using the standard addition method with rabbit liver MT I+II (Sigma). Results are expressed as µg g⁻¹ wet weight ~~(w.w.)~~ of tissue.

2.3. Metal analysis

Samples of 0.1-0.5 g wet weight of liver and muscle were digested in concentrated nitric acid 65% (Baker~~;~~) at room temperature overnight₁ and were heated at 80°C for 2 h. In order to determine the trace amounts of metal contained in the feed, samples of commercial pellets were also digested (n=3). Within each digestion series, appropriate blanks with ultra-pure water were also subjected to the same procedure in order to ~~account for~~ take background

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contamination levels into account. After cooling, solutions were transferred to a standard volume with ultra-pure water. Determination of metals (Cd, Cu, Zn, Fe, Pb, Hg and Mn) was undertaken using an ICP-Mass (Elan DRC-I, Perkin-Elmer Sciex). Samples of similar weight of certified reference material (DOLT-3 and LUTS-1, National Research Council of Canada, Ottawa), were digested and analyzed during each analytical run. The values of all elements found were in good agreement with the certified values, with the recoveries ranging from 91% to 104%.

2.4. Data analysis and calculations

Metal content ratio between muscle and liver was calculated in order to detect changes in the distribution of metals as a consequence of the thermal experimental conditions, and to show/reveal the proportion of accumulated metal in each tissue.

Bioaccumulation factor (BAF) was calculated for each metal-tissue-temperature combination in order to detect the effects of temperature on the global toxicokinetics among metals. #This was calculated as a quotient between the metal concentration in each tissue and the metal content in the commercial pellets as described by Dabrowska et al. (1996).

2.5. Statistical analysis

Statistical analyses were carried out using the software Stata 10 (Stata Corp). Transformations of the data were performed when the assumption of normality of residuals were/was not met. One-way ANOVA was used in the analysis of data to check the influence of temperature in/on each variable. The Scheffe test

was used as a post hoc test to ~~provedemonstrate~~ the differences among the three temperature groups. Two-way ANOVA, with temperature and metal as fixed factors, was used in the statistical analysis of BAFs, followed by a post hoc Bonferroni test. The Kruskal Wallis test was used when transformed data did not present homoscedasticity. Pearson correlation coefficients (r) were calculated between MT levels and metal content in liver, in order to measure the strength of association between these variables. Results are presented as means $\pm$ SEM and a p value lower than 0.05 was considered as statistically significant.

3. Results

3.1. Metal content in food and biometry

Metal contents of food pellets were $0.37838 \pm 0.05 \mu\text{g g}^{-1}$ w.w. for Cd, $11.636 \pm 0.747 \mu\text{g g}^{-1}$ w.w. for Cu, $172.64 \pm 8.89173 \pm 9 \mu\text{g g}^{-1}$ w.w. for Fe, $40.14 \pm 2.78 \pm 3 \mu\text{g g}^{-1}$ w.w. for Mn, $0.05205 \pm 0.01 \mu\text{g g}^{-1}$ w.w. for Hg, $0.15 \pm 0.09 \mu\text{g g}^{-1}$ w.w. for Pb and $138.94 \pm 8.76139 \pm 9 \mu\text{g g}^{-1}$ w.w. for Zn.

The weight and length ~~as well as~~ together with the condition index calculated for gilthead sea bream exposed to three experimental temperatures for three months are shown in Table 1. As can be seen, temperature has no effect on the condition index ~~and/or~~ weight, ~~in~~. In contrast, fish kept at 30°C (p<0.05) ~~was~~ were found to be significantly shorter than those kept at 22°C and 27°C.

Insert Table 1

3.2. Temperature effect on ~~metals content~~ metal contents and their distribution ~~between~~ among tissues

Metal content ~~achieved~~reached in liver and muscle of gilthead sea bream is shown in Figure 1. The ANOVA test shows a significant effect of temperature on Cd, Hg and Pb content ($p<0.001$), and on concentration of the essential metals Cu ($p<0.01$), Zn and Fe ($p<0.001$) in liver, but not on Mn content. Moreover, the results show a significant effect of temperature on Mn, Fe and Zn content ($p<0.01$) in muscle.

The muscle/liver ratios for each metal and temperature were calculated when possible and are shown in Table 2. They indicate the distribution of metals after temperature exposure and the proportion of each ~~one~~ in muscle in relation to liver. The highest muscle/liver ratio for fish maintained at 22 and 27 °C was reached by Hg; ~~however, whereas~~ at 30 °C the highest ratio was reached by Mn. As can be seen in Table 2, calculated ratios for Hg and Fe decreased significantly when temperature rose. Notably, and unlike for the other metals, Mn ratio rose significantly with increasing temperature.

Insert Figure 1

Insert Table 2

3.3. Temperature effect on toxicokinetic related processes: Bioaccumulation

Factor and MT induction

The BAF values obtained for each metal-tissue-temperature combination are shown in Table 3. The two-way ANOVA test shows significant differences among between metals in muscle ($p<0.001$) at all the ~~temperature~~temperatures tested. Hg BAF in this tissue is considerably higher in comparison with that calculated for the other measured metals, reaching a value of 0.45 at 30°C. This

means that muscle presents Hg content of almost a half of the feed content. In contrast, Mn BAF ~~exhibits~~ exhibits the lowest BAF ~~for~~ all temperature exposures. Statistical analysis of BAFs also showed significant differences among metals in liver ($p < 0.001$). BAF for Hg in liver differed from the BAFs for other metals at 27°C and 30°C, and reached the highest BAF registered in this study (3.20 at 30°C). It should be noted that Hg BAFs in liver at 27°C and 30°C are, respectively, nearly 3 and 5 fold higher than those determined at 22°C-~~respectively~~. A similar pattern to Hg was found for Cd, and in this case BAFs are 3 and 4 fold higher at 27°C and 30°C, respectively, than those determined at 22°C. ~~On the other hand~~, Mn BAFs in liver are the lowest among the studied metals.

Insert Table 3

MT levels determined in liver for the three experimental temperature groups are shown in Figure 1. As can be seen, there is a significant thermal effect on hepatic MT content. ~~A~~ MT significant induction is found in fish exposed to the ~~higher~~ highest temperature ($p < 0.001$). Furthermore, significant positive ~~correlation~~ correlations between MT and Cd ~~in liver~~ ($r = 0.52$; $p < 0.05$), Hg ($r = 0.78$; $p < 0.001$), Zn ($r = 0.82$; $p < 0.001$), Cu ($r = 0.82$; $p < 0.001$) and Fe ($r = 0.78$; $p < 0.001$) ~~in liver~~ have been found, but not with Pb and Mn.

4. Discussion

4.1. Metal content in food and biometry

Length differed significantly between animals exposed to 22°C and 30°C (Table 1), which could be due to an increase in energetic demand related to an increase in metabolic rate. A reduced growth and length at high temperature

was reported in *Oncorhynchus kisutch*, as a consequence of the energetic deficit after an elevated metabolism (Bowen et al., 2006).

Metal content of food pellets was in the same range as most of the values reported in the literature for commercial pellets (Ciardullo et al., 2008; Dang and Wang, 2009; Mackee et al., 2008; Minganti et al., 2010). Cd, Pb and Hg feed content are also below the established maximum permitted level in fish feed according to ~~the~~ EU Directive 2002/32/EC and 2005/87/EC amending Annex I to Directive 2002/32/EC.

4.2. Effect of temperature on metal content and its distribution ~~between~~among tissues

Metal contents in tissues (Figure 1) were in the same range as most of the values reported in the literature for farmed gilthead sea bream (Creti et al., 2010; Minghetti et al., 2008). It should be noted that Cd, Pb and Hg concentrations measured in muscle of *Sparus aurata* in the present work are below the established maximum permitted level in muscle of several species according to ~~the~~ EU Regulation (EC) No 1881/2006. We have found a general trend of increasing metal content in gilthead sea bream tissues with increasing temperatures. The positive relationship between temperature and increased metal uptake in different aquatic organisms, has been explained by accelerated biochemical and physiological processes, changes in the membrane permeability, ~~and~~ alterations of metal transport systems, and binding to several ligands (Baykan et al., 2007, Chowdhury and Blust, 2001; Jezierska and Witeska, 2006; Lemus and Chung, 1999).

The significant increase in Hg content in liver at both 27°C and 30°C ~~comparing~~ compared to at 22 °C, indicates strong temperature dependence. ~~Also~~ Additionally, the muscle/liver ratio (Table 2) confirms the preferential affinity of Hg to lipidic tissues such as the liver. Using an experimental trophic chain, Boudou et al. (1979) demonstrated that raising the temperature of the environment has a synergic effect on the quantities of Hg bioaccumulated by a carnivorous fish.

Cd content in liver also increased with rising temperature. Liver, as is well known, is an organ involved in storage and detoxification of Cd, and a progressive accumulation of dietary Cd over time has been explained by the continuous transference via the portal system from the digestive tract to the liver (Handy, 1993). In previous temperature effect studies in fish, the metal was supplied as waterborne Cd (Douben, 1989; Köck et al., 1996) and the temperature-dependent increase of metal content was explained by a greater increase in uptake rate than the increase found in the elimination rate (Douben, 1989). ~~Although~~, ~~although~~ mechanisms involved in Cd accumulation may differ into a great extent depending ~~of~~ on the Cd exposure source.

The highest Pb content in liver was reached by fish kept at 27°C. In some studies Pb uptake rate ~~was~~ ~~has been~~ affirmed to be temperature-dependent in fish (Köck et al., 1996).

An enhancement of Mn in muscle of fish maintained at 30°C in relation to those kept at colder temperatures was determined, nevertheless no significant variation in Mn was found in liver. However, when muscle/liver ratios were calculated, a significant effect of the temperature was detected in such a way that the ratio was higher at the warmer temperature, with the muscle acting as

aan Mn receptor tissue as temperature increased. However, little information is available concerning the effect of temperature on Mn accumulation in fish muscle. Adam et al. (1997) described two groups of organs with different elimination kinetics of ^{54}Mn , the first was the penetration or transit group, and the second the receptor or storage organs. According to this, liver was classified as aan Mn distribution and elimination tissue, and muscle as a receptor-one. The high levels of Cu in liver fromof fish kept at 30°C in comparison to those kept at lower temperatures are in agreement with findings reported in several studies. For instance, *Petenia kraussii* juveniles exposed to 22°C had lower Cu basal concentrations than those exposed to 30°C (Lemus and Chung, 1999). Temperature-related increase in Cu content has been explained by other authors as a consequence of a different extent in the rising of accumulation and elimination rates, resulting in a final positive balance for uptake (Glover et al., 2003; Lemus and Chung, 1999). In concordance with our results on Zn accumulation, Karakoç and Dinçer (2003) reported a temperature induced accumulation of Zn in liver, and Van Campenhout et al. (2007) described a promotion of Zn assimilation by temperature in fish. Also, Zn uptake in the apical cell membrane of rainbow trout intestine was also found to be a temperature-dependent process (Glover et al., 2003). In addition to the above findings, and taking into account the enhancement of Zn in muscle and liver of fish maintained at 30°C, and that no significant variation in the muscle/liver ratios was found in our study, we can suggest that a temperature-induced disturbance of Zn homeostasis in *Sparus aurata* may be occurring.

Levels of Fe in muscle and liver remained ~~quite~~fairly constant when temperature increased from 22°C to 27°C, as can be expected for a tightly regulated metal under physiological conditions. The sharp increase in Fe content in both tissues, and the different ratio value found at 30°C may be due to Fe homeostasis disturbance, indicating that some pathology is occurring. It should be born in mind that Fe loading leads to free radical damage by the Fenton reaction. Evidence in this direction has been found in fish and rats, suggesting a role for Fe in hepatic injury following hyperthermia. (Bloomer et al., 2008; Bowen et al., 2006; Skibba and Gwartney, 1997).

~~Summarizing~~In summary, essential metals in muscle are strongly regulated under physiological conditions, ~~however,~~although at 30°C, their homeostasis is disturbed as a result of a warmer temperature. The inverse relation ~~among~~between muscle/liver ratio and temperature is a general trend for all the metals studied, except Mn, which indicates that warmer temperatures facilitate metal regulation processes, in such a way that there is a preferential accumulation in liver thus protecting peripheral tissues.

4.3. Temperature effect on toxicokinetic related processes: Bioaccumulation

Factor and MT induction

BAF, the quotient between the metal concentration in tissue and the metal content in the feed, allowed us to quantify the accumulation of metals from feed in *Sparus aurata*, and to compare the temperature effect on bioaccumulation among the determined metals.

Metabolic rates of ectothermic organisms may be strongly dependent on temperature, and the same can be said for heavy ~~metal~~metals complexing with cellular constituents, which may affect tissue distribution and BAF. We can

suggest that variations in toxicokinetics caused by temperature exist in some of the studied ~~metal~~metals and tissues, although we cannot determine ~~if~~whether these changes are a consequence of the temperature effect on assimilation efficiency, or on feeding rate.

Hg presents the highest BAF values among metals for both tissues, at all temperatures tested (Table 3). Hg accumulation depends on its speciation, mainly because methylated Hg (MeHg) is better absorbed from the gastrointestinal tract than the inorganic form in such a way that in mammals 95% is absorbed (Liu et al., 2008). ~~Furthermore,~~ and furthermore it undergoes extensive enterohepatic recycling (Liu et al., 2008). Taking ~~in~~into account all the above, together with the fact that MeHg is the predominant form of Hg in fish feed (>80%) (Amlund et al., 2007), it is easy to understand the BAF values found for Hg.

In agreement with hepatic Cd content, BAF values found in liver are higher at warmer temperatures and, as discussed above, this may be due to the function that this organ has in storage and detoxification of Cd (Jezierska and Witeska, 2006; Köck et al., 1996; Yang and Chen, 1996).

BAF for Pb in liver is lower than the BAF for the other two toxic metals (Cd and Hg), which ~~could~~might be due to Pb preferential deposition into internal soft tissues other than liver as reported in *Oncorhynchus mykiss* (Alves et al., 2006).

Mn shares a large number of transport mechanisms with other metals, ~~like~~ such as transferrin, or the promiscuous divalent metal transporter (DMT) (Bury and Grosell, 2003), and excretion mechanisms such as bile (Papavasiliou et al., 1966), and for this reason, similar Mn behavior to other metals should be expected. However, in this study Mn has a completely different reaction and

presents the lowest uptake from dietary sources, as the BAF values suggestshow. This may mean that Mn storesis stored in other tissues than liver, and this phenomenon may be amplified with increasing temperature.

It is important to note that BAFBAFs of Cu and Zn in liver are almost the same when determined for the same temperature, which indicates that a similar fraction of metal contained in feed is retained, and also that temperature has a similar effect on the overall metabolism of both elements.

Essential metals have transport systems that include severalboth specific and general mechanisms. They are strongly regulated in organisms under non-stressful conditions, and consequently environmental factor variations should not greatly affect their concentration. On the other hand, toxic metals have no specific mechanisms and use essential element transport proteins and channels, such as divalent metal transporter (DMT1), diverse ZIP proteins or MT (Bury et al., 2003; Coyle et al., 2002; He et al., 2006; Vesey et al., 2010). It is clear that the common mechanisms may be affected by temperature to a different degree or in a different measure-or way depending on the metal and tissue. For instance, the temperature-dependent increase of membrane fluidity and physical diffusion rate may be of special relevance for Hg accumulation (Foulkes, 2000). It is difficult to determine at which level the mechanisms implicated in the toxicokinetics of each metal are influenced by temperature.

The effect of temperature on MT level in liver could be a direct thermal response, or may be related to the increase in metal content. MT synthesis is considered one of the best-known biochemical detoxification mechanisms for metal and it is widely demonstrated that its induction may be influenced by metal contamination. It is very common to try to relate seasonal variability to

temperature, but in this case the reproductive state may also be involved (Gorbi et al., 2005; Köck et al., 1996; Olsson et al., 1996; Rotchell et al., 2001). There are only a few studies specially designed for elucidating the effect of temperature on MT synthesis in fish. Van Cleef-Toedt et al. (2001) demonstrated that non-spawning *Fundulus heteroclitus* exposed to thermal stress exhibited significantly elevated liver, gill, and intestine MT mRNA expression compared with controls.

In this study we have found an unusual correlation between MT and Fe content in liver of *Sparus aurata*. MT is known to be a protein involved in protection against oxidative stress (Viarengo et al 1999; Andrews, 2000; Gourgou et al. 2010), including heat-induced oxidative stress (Ivanina et al., 2009). On the other hand, Fe may play a role in hepatic injury after hyperthermia and is considered as an indicator of liver damage in mammals (Bloomer et al., 2008; Bowen et al., 2006; Skibba and Gwartney, 1997). Fe excess is believed to generate oxidative stress, understood as an increase in the steady state concentration of oxygen radical intermediates (Puntarulo, 2005). Taking all the above together we can suggest that the MT-Fe correlation may be due to the accumulation of Fe in liver produced by hyperthermia, which can cause oxidative stress which, in turn, produces MT induction .

5. Conclusion

The results obtained in this study show that metal bioaccumulation from non-experimentalexperimentally contaminated commercial feed and MT induction takes place at warm temperatures in *Sparus aurata*. Furthermore, different patterns of metal distribution among tissues and temperature indicate disturbed

essential metal homeostasis and higher levels of toxic metal bioaccumulation, particularly of Hg and Cd. The interactions among water temperature, metal uptake from feed and their distribution between tissues in these species exist, but we can still ~~we cannot~~not provide a full mechanistic explanation for ~~it~~this. Future research on the identification of the temperature-dependent mechanisms involved in metal toxicokinetics is required in order to reach a better understanding of temperature role in ecotoxicology and its implications in aquaculture in a warming world context.

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562

- Warming temperatures has an influence on MT in liver and on metal accumulation in liver and muscle in gilthead sea bream.
- The effect of temperature on accumulation differs among metals, being cadmium and mercury accumulation the most sensitive to this variation.
- Essential metal homeostasis was disturbed at the warmest temperature.
- MT was correlated with cadmium, cooper, mercury, zinc and iron content in liver.

1 **Long-term effect of temperature on bioaccumulation of dietary metals and**
2 **metallothionein induction in *Sparus aurata*.**

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12 **Abstract**

13 Previous studies have demonstrated that the commercial feed of aquacultured
14 fish contains trace amounts of toxic and essential metals which can accumulate
15 in tissues and finally be ingested by consumers. Recently rising temperatures,
16 associated to the global warming phenomenon, have been reported as a factor
17 to be taken into consideration in ecotoxicology, since temperature-dependent
18 alterations in bioavailability, toxicokinetics and biotransformation rates can be
19 expected. *Sparus aurata* were kept at 22°C, 27°C and 30°C for three months in
20 order to determine the temperature effect on metallothionein induction and
21 metal bioaccumulation from a non-experimentally contaminated commercial
22 feed. A significant temperature-dependent accumulation of cadmium (Cd),
23 copper (Cu), mercury (Hg), zinc (Zn), lead (Pb) and iron (Fe) was found in liver,
24 together with that of manganese (Mn), Fe and Zn in muscle. Hg presented the

25 highest bioaccumulation factor, and essential metal homeostasis was disturbed
26 in both tissues at warm temperatures. An enhancement of hepatic
27 metallothionein induction was found in fish exposed to the highest temperature.

28 **Keywords:** metal; metallothionein; bioaccumulation factor; temperature; *Sparus*
29 *aurata*

1. Introduction

The aquatic ecosystems are undergoing a warming in their deep and surface waters, a fact which may have significant consequences on the organisms inhabiting them (Noyes et al., 2009). Gilthead sea bream *Sparus aurata* is a cosmopolitan species distributed throughout the Mediterranean and the NE Atlantic, and which is of great economic interest since it is one of the principal species of Mediterranean aquaculture. Being an ectothermic species, it is vulnerable to the effects of temperature variations on its metabolism and physiology. These variations may produce changes in the toxicokinetics, bioavailability, biotransformation, homeostasis, absorption rate and elimination of different compounds (Douben, 1989; Köck et al., 1996; Yang and Chen, 1996). Other key physiological mechanisms such as respiration, feeding rate, growth and reproduction may also be affected (Bowen et al., 2006; Heugens et al., 2001). The thermic induced variations in the toxicokinetics of pollutants, together with the increase in exposure to the same as a consequence of climate warming (Carrie et al., 2010) may present a risk for the development and survival of species of commercial interest, affecting the quality of the end product (Noyes et al., 2009).

In cultured fish, it has been demonstrated that the feed is the principal source of contamination by metals Cadmium (Cd), lead (Pb) and mercury (Hg), among others, are potentially toxic and tend to accumulate in the tissues, which in the end are ingested by the consumers (Fernandes et al., 2009; Maule et al., 2007; Cretì et al., 2010). Their accumulation in the organisms depends on the concentration, route of absorption, environmental conditions and other intrinsic

factors (Bowen et al., 2006; Chowdhury and Blust, 2001; Jezierska and Witeska, 2006; Karakoç and Dinçer, 2003; Lemus and Chung, 1999).

Due to the growth in the activity of aquaculture in recent decades, it has become of special relevance to learn the influence of the increase in temperature on the possible routes of absorption, accumulation and elimination of metals in these organisms in the context of global warming. Temperature may be a determining factor in the capture, transporting and metabolism of the metals incorporated through the feed, both of the essential metals which may become toxic at high concentrations in the tissues, and of the non-essential metals.

Metallothionein (MT) is a low-molecular-weight metal binding protein and is known to play an important role in protection against heavy metal toxicity. In addition to the detoxification of toxic metals such as Cd and Hg, MT is involved in the maintenance of homeostasis of essential trace elements such as zinc (Zn) and copper (Cu) (Coyle et al., 2002; Hamilton and Mehrle, 1986). Its role in the protection against xenobiotics or in the cellular protection against oxidative stress should be underlined (Coyle et al., 2002; Van Cleef-Toedt et al., 2001).

Although its synthesis is related to the metal exposure, its levels can be affected by endogenous and exogenous factors such as the reproductive cycle or the temperature (Van Cleef-Toedt et al., 2001). Variations in the water temperature could directly or indirectly modify the behavior of this protein as regards the bioaccumulation of metals, as well as its participation in toxicokinetic processes (Baykan et al., 2007; Gorbi et al., 2005; Rotchell et al., 2001).

Given the lack of information as regards the effect of temperature on the bioaccumulation of metals via dietary sources, and on the synthesis of MT, an

understanding of these processes in the light of the problem of global warming and its repercussions on species of commercial interest such as *Sparus aurata* is required. As such, the aims of this work are:

1) To determine the effect of temperature on the bioaccumulation of essential (Cu, Fe, Mn and Zn) and non-essential (Cd, Hg and Pb) metals experimentally via a non-contaminated commercial feed.

2) To discover whether temperature has any influence on the homeostasis of the essential metals.

3) To elucidate the role of metallothionein in the above mentioned temperature induced changes.

2. Materials and Methods

2.1. Animal collection and maintenance

Adult *Sparus aurata* were distributed and acclimated in 500 L tanks containing seawater (37‰) at a constant temperature of 22°C, with continuous aeration and natural photoperiod in a closed circuit for two months prior to the experiment. Subsequently, the temperature of two of the experimental groups was gradually increased to reach 27°C and 30°C respectively. Control groups of fish were maintained at 22 °C throughout the experiments. The animals were fed with commercial pellets (1.5% of body mass per day) and the survival percentage was 99%. Fish were kept under constant conditions for three months. After this period, 6-7 fish were removed at random from each experimental group and placed in water containing 30 mg L⁻¹ of anesthetic clove oil. Lengths and weights of gilthead sea bream were recorded. Fish were sacrificed; livers and a piece of dorsal muscle tissue were dissected and immediately frozen in liquid nitrogen and stored at -80 °C.

2.2. MT determination by differential pulse polarography

Approximately 0.2 g wet weight portions of frozen liver were homogenized using ultra-turrax in 20 mM Tris-HCl buffer, 1 mM DTT and 0.2 mM PMSF pH 8.6 in an ice bath. The homogenates were centrifuged at 30 000 g for 45 min at 4°C. The supernatant was heated at 80°C for 10 min in order to denature high molecular weight proteins and subsequently centrifuged at 30 000 g for 45 min at 4°C. The heat-treated supernatant, containing thermally stable MT, was separated from precipitated proteins. MT was measured using differential pulse polarography (Ureña et al., 2007).

An aliquot of the heat-treated supernatant was added to the polarographic cell, containing 20 mL hexammincobalt chloride buffer (the supporting electrolyte), together with Triton-X (0.025% v/v). The cell was purged for 2 min with purified N₂ prior to analysis. The polarographic response was measured during a potential scan between -1.38 V and -1.7 V (Model 757 VA Computrace Analyser, Methrom, Switzerland) in SMDE mode. Quantification of MT was performed by using the standard addition method with rabbit liver MT I+II (Sigma). Results are expressed as $\mu\text{g g}^{-1}$ wet weight of tissue.

2.3. Metal analysis

Samples of 0.1-0.5 g wet weight of liver and muscle were digested in concentrated nitric acid 65% (Baker) at room temperature overnight, and were heated at 80°C for 2 h. In order to determine the trace amounts of metal contained in the feed, samples of commercial pellets were also digested (n=3). Within each digestion series, appropriate blanks with ultra-pure water were also subjected to the same procedure in order to take background contamination

levels into account. After cooling, solutions were transferred to a standard volume with ultra-pure water. Determination of metals (Cd, Cu, Zn, Fe, Pb, Hg and Mn) was undertaken using an ICP-Mass (Elan DRC-I, Perkin-Elmer Sciex). Samples of similar weight of certified reference material (DOLT-3 and LUTS-1, National Research Council of Canada, Ottawa), were digested and analyzed during each analytical run. The values of all elements found were in good agreement with the certified values, with the recoveries ranging from 91% to 104%.

2.4. Data analysis and calculations

Metal content ratio between muscle and liver was calculated in order to detect changes in the distribution of metals as a consequence of the thermal experimental conditions, and to reveal the proportion of accumulated metal in each tissue.

Bioaccumulation factor (BAF) was calculated for each metal-tissue-temperature combination in order to detect the effects of temperature on the global toxicokinetics among metals. This was calculated as a quotient between the metal concentration in each tissue and the metal content in the commercial pellets as described by Dabrowska et al. (1996).

2.5. Statistical analysis

Statistical analyses were carried out using the software Stata 10 (Stata Corp). Transformations of the data were performed when the assumption of normality of residuals was not met. One-way ANOVA was used in the analysis of data to check the influence of temperature on each variable. The Scheffe test was used

as a post hoc test to demonstrate the differences among the three temperature groups. Two-way ANOVA, with temperature and metal as fixed factors, was used in the statistical analysis of BAFs, followed by a post hoc Bonferroni test. The Kruskal Wallis test was used when transformed data did not present homoscedasticity. Pearson correlation coefficients (r) were calculated between MT levels and metal content in liver, in order to measure the strength of association between these variables. Results are presented as means $\pm$ SEM and a p value lower than 0.05 was considered as statistically significant.

3. Results

3.1. Metal content in food and biometry

Metal contents of food pellets were $0.38 \pm 0.05 \mu\text{g g}^{-1}$ w.w. for Cd, $11.6 \pm 0.7 \mu\text{g g}^{-1}$ w.w. for Cu, $173 \pm 9 \mu\text{g g}^{-1}$ w.w. for Fe, $40. \pm 3 \mu\text{g g}^{-1}$ w.w. for Mn, $0.05 \pm 0.01 \mu\text{g g}^{-1}$ w.w. for Hg, $0.15 \pm 0.09 \mu\text{g g}^{-1}$ w.w. for Pb and $139 \pm 9 \mu\text{g g}^{-1}$ w.w. for Zn.

The weight and length together with the condition index calculated for gilthead sea bream exposed to three experimental temperatures for three months are shown in Table 1. As can be seen, temperature has no effect on the condition index or weight. In contrast, fish kept at 30°C ($p < 0.05$) were found to be significantly shorter than those kept at 22°C and 27°C.

Insert Table 1

3.2. Temperature effect on metal contents and their distribution among tissues

Metal content reached in liver and muscle of gilthead sea bream is shown in Figure 1. The ANOVA test shows a significant effect of temperature on Cd, Hg

and Pb content ($p<0.001$), and on concentration of the essential metals Cu ($p<0.01$), Zn and Fe ($p<0.001$) in liver, but not on Mn content. Moreover, the results show a significant effect of temperature on Mn, Fe and Zn content ($p<0.01$) in muscle.

The muscle/liver ratios for each metal and temperature were calculated when possible and are shown in Table 2. They indicate the distribution of metals after temperature exposure and the proportion of each in muscle in relation to liver. The highest muscle/liver ratio for fish maintained at 22 and 27 °C was reached by Hg, whereas at 30 °C the highest ratio was reached by Mn. As can be seen in Table 2, calculated ratios for Hg and Fe decreased significantly when temperature rose. Notably, and unlike for the other metals, Mn ratio rose significantly with increasing temperature.

Insert Figure 1

Insert Table 2

3.3. Temperature effect on toxicokinetic related processes: Bioaccumulation Factor and MT induction

The BAF values obtained for each metal-tissue-temperature combination are shown in Table 3. The two-way ANOVA test shows significant differences among between metals in muscle ($p<0.001$) at all the temperatures tested. Hg BAF in this tissue is considerably higher in comparison with that calculated for the other measured metals, reaching a value of 0.45 at 30°C. This means that muscle presents Hg content of almost a half of the feed content. In contrast, Mn BAF exhibits the lowest BAF for all temperature exposures.

Statistical analysis of BAFs also showed significant differences among metals in liver ($p < 0.001$). BAF for Hg in liver differed from the BAFs for other metals at 27°C and 30°C, and reached the highest BAF registered in this study (3.20 at 30°C). It should be noted that Hg BAFs in liver at 27°C and 30°C are, respectively, nearly 3 and 5 fold higher than those determined at 22°C. A similar pattern to Hg was found for Cd, and in this case BAFs are 3 and 4 fold higher at 27°C and 30°C, respectively, than those determined at 22°C. Mn BAFs in liver are the lowest among the studied metals.

Insert Table 3

MT levels determined in liver for the three experimental temperature groups are shown in Figure 1. As can be seen, there is a significant thermal effect on hepatic MT content. An MT significant induction is found in fish exposed to the highest temperature ($p < 0.001$). Furthermore, significant positive correlations between MT and Cd in liver ($r = 0.52$; $p < 0.05$), Hg ($r = 0.78$; $p < 0.001$), Zn ($r = 0.82$; $p < 0.001$), Cu ($r = 0.82$; $p < 0.001$) and Fe ($r = 0.78$; $p < 0.001$) have been found, but not with Pb and Mn.

4. Discussion

4.1. Metal content in food and biometry

Length differed significantly between animals exposed to 22°C and 30°C (Table 1), which could be due to an increase in energetic demand related to an increase in metabolic rate. A reduced growth and length at high temperature was reported in *Oncorhynchus kisutch*, as a consequence of the energetic deficit after an elevated metabolism (Bowen et al., 2006).

Metal content of food pellets was in the same range as most of the values reported in the literature for commercial pellets (Ciardullo et al., 2008; Dang and Wang, 2009; Mackee et al., 2008; Minganti et al., 2010). Cd, Pb and Hg feed content are also below the established maximum permitted level in fish feed according to EU Directive 2002/32/EC and 2005/87/EC amending Annex I to Directive 2002/32/EC.

4.2. Effect of temperature on metal content and its distribution among tissues

Metal contents in tissues (Figure 1) were in the same range as most of the values reported in the literature for farmed gilthead sea bream (Creti et al., 2010; Minghetti et al., 2008). It should be noted that Cd, Pb and Hg concentrations measured in muscle of *Sparus aurata* in the present work are below the established maximum permitted level in muscle of several species according to EU Regulation (EC) No 1881/2006. We have found a general trend of increasing metal content in gilthead sea bream tissues with increasing temperatures. The positive relationship between temperature and increased metal uptake in different aquatic organisms has been explained by accelerated biochemical and physiological processes, changes in the membrane permeability, alterations of metal transport systems, and binding to several ligands (Baykan et al., 2007, Chowdhury and Blust, 2001; Jezierska and Witeska, 2006; Lemus and Chung, 1999).

The significant increase in Hg content in liver at both 27°C and 30°C compared to at 22 °C indicates strong temperature dependence. Additionally, the muscle/liver ratio (Table 2) confirms the preferential affinity of Hg to lipidic

tissues such as the liver. Using an experimental trophic chain, Boudou et al. (1979) demonstrated that raising the temperature of the environment has a synergic effect on the quantities of Hg bioaccumulated by a carnivorous fish. Cd content in liver also increased with rising temperature. Liver, as is well known, is an organ involved in storage and detoxification of Cd, and a progressive accumulation of dietary Cd over time has been explained by the continuous transference via the portal system from the digestive tract to the liver (Handy, 1993). In previous temperature effect studies in fish, the metal was supplied as waterborne Cd (Douben, 1989; Köck et al., 1996) and the temperature-dependent increase of metal content was explained by a greater increase in uptake rate than the increase found in the elimination rate (Douben, 1989), although mechanisms involved in Cd accumulation may differ to a great extent depending on the Cd exposure source.

The highest Pb content in liver was reached by fish kept at 27°C. In some studies Pb uptake rate has been affirmed to be temperature-dependent in fish (Köck et al., 1996).

An enhancement of Mn in muscle of fish maintained at 30°C in relation to those kept at colder temperatures was determined, nevertheless no significant variation in Mn was found in liver. However, when muscle/liver ratios were calculated, a significant effect of the temperature was detected in such a way that the ratio was higher at the warmer temperature, with the muscle acting as an Mn receptor tissue as temperature increased. However, little information is available concerning the effect of temperature on Mn accumulation in fish muscle. Adam et al. (1997) described two groups of organs with different elimination kinetics of ⁵⁴Mn, the first was the penetration or transit group, and

the second the receptor or storage organs. According to this, liver was classified as an Mn distribution and elimination tissue, and muscle as a receptor. The high levels of Cu in liver of fish kept at 30°C in comparison to those kept at lower temperatures are in agreement with findings reported in several studies. For instance, *Petenia kraussii* juveniles exposed to 22°C had lower Cu basal concentrations than those exposed to 30°C (Lemus and Chung, 1999). Temperature-related increase in Cu content has been explained by other authors as a consequence of a different extent in the rising of accumulation and elimination rates, resulting in a final positive balance for uptake (Glover et al., 2003; Lemus and Chung, 1999).

In concordance with our results on Zn accumulation, Karakoç and Dinçer (2003) reported a temperature induced accumulation of Zn in liver, and Van Campenhout et al. (2007) described a promotion of Zn assimilation by temperature in fish. Zn uptake in the apical cell membrane of rainbow trout intestine was also found to be a temperature-dependent process (Glover et al., 2003). In addition to the above findings, and taking into account the enhancement of Zn in muscle and liver of fish maintained at 30°C, and that no significant variation in the muscle/liver ratios was found in our study, we can suggest that a temperature-induced disturbance of Zn homeostasis in *Sparus aurata* may be occurring.

Levels of Fe in muscle and liver remained fairly constant when temperature increased from 22°C to 27°C, as can be expected for a tightly regulated metal under physiological conditions. The sharp increase in Fe content in both tissues, and the different ratio value found at 30°C may be due to Fe homeostasis disturbance, indicating that some pathology is occurring. It should

be born in mind that Fe loading leads to free radical damage by the Fenton reaction. Evidence in this direction has been found in fish and rats, suggesting a role for Fe in hepatic injury following hyperthermia. (Bloomer et al., 2008; Bowen et al., 2006; Skibba and Gwartney, 1997).

In summary, essential metals in muscle are strongly regulated under physiological conditions, although at 30°C their homeostasis is disturbed as a result of a warmer temperature. The inverse relation between muscle/liver ratio and temperature is a general trend for all the metals studied except Mn, which indicates that warmer temperatures facilitate metal regulation processes in such a way that there is a preferential accumulation in liver thus protecting peripheral tissues.

4.3. Temperature effect on toxicokinetic related processes: Bioaccumulation Factor and MT induction

BAF, the quotient between the metal concentration in tissue and the metal content in the feed, allowed us to quantify the accumulation of metals from feed in *Sparus aurata*, and to compare the temperature effect on bioaccumulation among the determined metals.

Metabolic rates of ectothermic organisms may be strongly dependent on temperature, and the same can be said for heavy metals complexing with cellular constituents, which may affect tissue distribution and BAF. We can suggest that variations in toxicokinetics caused by temperature exist in some of the studied metals and tissues, although we cannot determine whether these changes are a consequence of the temperature effect on assimilation efficiency, or on feeding rate.

Hg presents the highest BAF values among metals for both tissues at all temperatures tested (Table 3). Hg accumulation depends on its speciation, mainly because methylated Hg (MeHg) is better absorbed from the gastrointestinal tract than the inorganic form in such a way that in mammals 95% is absorbed (Liu et al., 2008), and furthermore it undergoes extensive enterohepatic recycling (Liu et al., 2008). Taking into account all the above, together with the fact that MeHg is the predominant form of Hg in fish feed (>80%) (Amlund et al., 2007), it is easy to understand the BAF values found for Hg.

In agreement with hepatic Cd content, BAF values found in liver are higher at warmer temperatures and, as discussed above, this may be due to the function that this organ has in storage and detoxification of Cd (Jezierska and Witeska, 2006; Köck et al., 1996; Yang and Chen, 1996).

BAF for Pb in liver is lower than the BAF for the other two toxic metals (Cd and Hg), which might be due to Pb preferential deposition into internal soft tissues other than liver as reported in *Oncorhynchus mykiss* (Alves et al., 2006).

Mn shares a large number of transport mechanisms with other metals such as transferrin, or the promiscuous divalent metal transporter (DMT) (Bury and Grosell, 2003), and excretion mechanisms such as bile (Papavasiliou et al., 1966), and for this reason similar Mn behavior to other metals should be expected. However, in this study Mn has a completely different reaction and presents the lowest uptake from dietary sources, as the BAF values show. This may mean that Mn is stored in other tissues than liver, and this phenomenon may be amplified with increasing temperature.

It is important to note that BAFs of Cu and Zn in liver are almost the same when determined for the same temperature, which indicates that a similar fraction of metal contained in feed is retained, and also that temperature has a similar effect on the overall metabolism of both elements.

Essential metals have transport systems that include both specific and general mechanisms. They are strongly regulated in organisms under non-stressful conditions, and consequently environmental factor variations should not greatly affect their concentration. On the other hand, toxic metals have no specific mechanisms and use essential element transport proteins and channels, such as divalent metal transporter (DMT1), diverse ZIP proteins or MT (Bury et al., 2003; Coyle et al., 2002; He et al., 2006; Vesey et al., 2010). It is clear that the common mechanisms may be affected by temperature to a different degree or in a different way depending on the metal and tissue. For instance, the temperature-dependent increase of membrane fluidity and physical diffusion rate may be of special relevance for Hg accumulation (Foulkes, 2000). It is difficult to determine at which level the mechanisms implicated in the toxicokinetics of each metal are influenced by temperature.

The effect of temperature on MT level in liver could be a direct thermal response, or may be related to the increase in metal content. MT synthesis is considered one of the best-known biochemical detoxification mechanisms for metal and it is widely demonstrated that its induction may be influenced by metal contamination. It is very common to try to relate seasonal variability to temperature, but in this case the reproductive state may also be involved (Gorbi et al., 2005; Köck et al., 1996; Olsson et al., 1996; Rotchell et al., 2001). There are only a few studies specially designed for elucidating the effect of

temperature on MT synthesis in fish. Van Cleef-Toedt et al. (2001) demonstrated that non-spawning *Fundulus heteroclitus* exposed to thermal stress exhibited significantly elevated liver, gill, and intestine MT mRNA expression compared with controls.

In this study we have found an unusual correlation between MT and Fe content in liver of *Sparus aurata*. MT is known to be a protein involved in protection against oxidative stress (Viarengo et al 1999; Andrews, 2000; Gourgou et al. 2010), including heat-induced oxidative stress (Ivanina et al., 2009). On the other hand, Fe may play a role in hepatic injury after hyperthermia and is considered as an indicator of liver damage in mammals (Bloomer et al., 2008; Bowen et al., 2006; Skibba and Gwartney, 1997). Fe excess is believed to generate oxidative stress, understood as an increase in the steady state concentration of oxygen radical intermediates (Puntarulo, 2005). Taking all the above together we can suggest that the MT-Fe correlation may be due to the accumulation of Fe in liver produced by hyperthermia, which can cause oxidative stress which, in turn, produces MT induction .

5. Conclusion

The results obtained in this study show that metal bioaccumulation from non-experimentally contaminated commercial feed and MT induction takes place at warm temperatures in *Sparus aurata*. Furthermore, different patterns of metal distribution among tissues and temperature indicate disturbed essential metal homeostasis and higher levels of toxic metal bioaccumulation, particularly of Hg and Cd. The interactions among water temperature, metal uptake from feed and their distribution between tissues in these species exist, but we can still not

provide a full mechanistic explanation for this. Future research on the identification of the temperature-dependent mechanisms involved in metal toxicokinetics is required in order to reach a better understanding of temperature role in ecotoxicology and its implications in aquaculture in a warming world context.

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Figure captions

Figure 1. MT levels in liver and metal content in muscle and liver of *Sparus aurata* exposed to three different experimental temperatures. Data represent mean $\pm$ SEM ($n \geq 6$). Means that do not share the same letter differ significantly ($p < 0.05$).

Figure 1
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Table 1. Biometry of *Sparus aurata* kept under temperature exposure for three months. Data represent mean $\pm$ SEM ($n \geq 6$). Means that do not share the same letter differ significantly ($p < 0.05$).

	22°C	27°C	30°C
Weigth (g)	290 $\pm$ 76 ^a	259 $\pm$ 56 ^a	218 $\pm$ 64 ^a
Lenght (cm)	234 $\pm$ 21 ^a	221 $\pm$ 22 ^{ab}	199 $\pm$ 19 ^b
CI (g cm ⁻³ *10 ⁻⁵)	2.2 $\pm$ 0.3 ^a	2.4 $\pm$ 0.5 ^a	2.7 $\pm$ 0.4 ^a

Table 2. Muscle/liver metal ratios calculated of *Sparus aurata* exposed long-term to three different experimental temperatures. Data represent mean $\pm$ SEM ($n \geq 6$). Means that do not share the same letter differ significantly ($p < 0.05$).

	22°C	27°C	30°C
Fe	0.09 $\pm$ 0.02 ^a	0.06 $\pm$ 0.01 ^a	0.07 $\pm$ 0.05 ^b
Mn	0.07 $\pm$ 0.04 ^a	0.11 $\pm$ 0.02 ^{ab}	0.25 $\pm$ 0.02 ^b
Hg	0.43 $\pm$ 0.23 ^a	0.16 $\pm$ 0.06 ^b	0.15 $\pm$ 0.09 ^b
Zn	0.14 $\pm$ 0.08 ^a	0.08 $\pm$ 0.04 ^a	0.10 $\pm$ 0.06 ^a

Table 3 Metal bioaccumulation factor (BAF) in liver and muscle of *Sparus aurata* kept under temperature exposure for three months. Data represent mean ± SEM (n≥6). Means that do not share the same letter at the same temperature differ significantly (p<0.05).

Tissue	T	Cd	Cu	Fe	Mn	Hg	Pb	Zn
Liver	22°C	0.35 ± 0.33 ^{ab}	0.19 ± 0.07 ^{ab}	0.16 ± 0.06 ^{ab}	0.03 ± 0.01 ^a	0.66 ± 0.33 ^b	0.14 ± 0.11 ^{ab}	0.20 ± 0.05 ^{ab}
	27°C	1.01 ± 0.23 ^a	0.31 ± 0.01 ^b	0.16 ± 0.01 ^b	0.03 ± 0.01 ^b	1.73 ± 0.57 ^c	0.43 ± 0.12 ^b	0.30 ± 0.08 ^b
	30°C	1.40 ± 0.46 ^a	0.61 ± 0.24 ^b	0.48 ± 0.11 ^{bc}	0.03 ± 0.01 ^c	3.20 ± 0.79 ^d	0.27 ± 0.11 ^{bc}	0.57 ± 0.07 ^{bc}
Muscle	22°C			0.017 ± 0.008 ^a	0.002 ± 0.001 ^a	0.27 ± 0.13 ^b		0.03 ± 0.01 ^a
	27°C			0.008 ± 0.003 ^a	0.003 ± 0.001 ^a	0.27 ± 0.06 ^b		0.02 ± 0.01 ^a
	30°C			0.04 ± 0.03 ^a	0.006 ± 0.004 ^a	0.45 ± 0.27 ^b		0.06 ± 0.03 ^a