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The expression "elevated temperatures" has been replaced by "temperature"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

1 **Long-term effect of ~~elevated~~ temperature on bioaccumulation of dietary**

2 **metals and 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 ~~on~~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

1

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.

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; Cretì et al., 2010). Their accumulation in the organisms depends on the concentration, route of absorption, environmental conditions and other

54 intrinsic factors (Bowen et al., 2006; Chowdhury and Blust, 2001; Jezierska and

55 Witeska, 2006; Karakoç and Dinçer, 2003; Lemus and Chung, 1999).

56 Due to the growth in the activity of aquaculture activity in recent decades, it has

57 become of special relevance to learn the influence of the increase in

58 temperature on the possible routes of absorption, accumulation and elimination

59 of metals in these organisms in the context of global warming. The

60 temperatureTemperature may be a determining factor in the capture,

61 transporting and metabolism of the metals incorporated through the feed, both

62 of the essential metals which may become toxic at high concentrations in the

63 tissues, and of the non-essential metals.

64 Metallothionein (MT) is a low-molecular-weight metal binding protein and is

65 known to play an important role in protection against heavy metal toxicity. In

66 addition to the detoxification of toxic metals such as Cd and Hg, MT is involved

67 in the maintenance of homeostasis of essential trace elements such as zinc

68 (Zn) and copper (Cu) (Coyle et al., 2002; Hamilton and Mehrle, 1986). Its role in

69 the protection against xenobiotics or in the cellular protection against oxidative

70 stress should be underlined (Coyle et al., 2002; Van Cleef-Toedt et al., 2001).

71 Although its synthesis is related to the metal exposure, its levels can be affected

72 by endogenous and exogenous factors such as the reproductive cycle or the

73 temperature (Van Cleef-Toedt et al., 2001). Variations in the water temperature

74 could modify, directly or indirectly, modify the behavior of this protein as regards

75 the bioaccumulation of metals, as well as its participation in toxicokinetic

76 processes (Baykan et al., 2007; Gorbi et al., 2005; Rotchell et al., 2001).

77 Given the lack of information as regards the effect of temperature on the

78 bioaccumulation of metals via dietary sources, and on the synthesis of MT, an

4

79 understanding of these processes in the light of the problem of global warming

80 and its repercussions on species of commercial interest such as *Sparus aurata*

81 is required. As such, the aims of this work are:

82 1) To determine the effect of temperature on the bioaccumulation of essential

83 (Cu, Fe, Mn and Zn) and non-essential (Cd, Hg and Pb) metals experimentally

84 via a non-contaminated commercial feed.

85 2) To discover whether ~~the~~ temperature has any influence on the homeostasis

86 of the essential metals.

87 3) To elucidate the role of metallothionein ~~role~~ in the above mentioned

88 temperature induced changes.

89 **2. Materials and Methods**

90 *2.1. Animal collection and maintenance*

91 Adult *Sparus aurata* were distributed and acclimated in 500 L tanks containing

92 seawater (37~~‰),~~‰) at a constant temperature of 22°C, with continuous aeration

93 and natural photoperiod~~,~~ in a closed circuit for two months ~~before starting~~prior to

94 the experiment. Subsequently, the temperature of two of the experimental

95 groups was gradually increased to reach 27°C and 30°C respectively. Control

96 groups of fish were maintained at 22 ºC throughout the experiments. The

97 animals were fed with commercial pellets (1.5% of body mass per day) and the

98 survival percentage was 99%. Fish were kept under constant conditions for

99 three months. After this period, 6-7 fish were removed at random from each

100 experimental group and ~~held~~placed in water containing 30 mg $L^{-1}$of anesthetic

101 clove oil. Lengths and weights of gilthead sea bream were recorded. Fish were

102 sacrificed; livers and a piece of dorsal muscle tissue were dissected and

103 immediately frozen in liquid nitrogen and stored at -80 °C.

104 *2.2. MT determination by differential pulse polarography*

105 Approximately 0.2 g wet weight portions of frozen liver were homogenized using

106 ultra-turrax in 20 mM Tris-HCl buffer, 1 mM DTT and 0.2 mM PMSF pH 8.6 in

107 an ice bath. The homogenates were centrifuged at 30 000 *g* for 45 min at 4°C.

108 The supernatant was heated at 80°C for 10 min in order to denature high

109 molecular weight proteins and subsequently centrifuged at 30 000 g for 45 min

110 at 4°C,The heat-treated supernatant, containing thermally stable MT, was

111 separated from precipitated proteins. MT was measured using differential pulse

112 polarography ~~as described by~~ (Ureña et al~~.,~~ 2007).

113 An aliquot of the heat-treated supernatant was added to the polarographic cell,

114 containing 20 mL hexammincobalt chloride buffer (the supporting electrolyte),

115 together with Triton-X (0.025% v/v). The cell was purged for 2 min with purified

116 N_2 prior to analysis. The polarographic response was measured during a

117 potential scan between -1.38 V and -1.7 V (Model 757 VA Computrace

118 Analyser, Methrom, Switzerland) in SMDE mode. Quantification of MT was

119 performed by using the standard addition method with rabbit liver MT I+II

120 (Sigma). Results are expressed as µg g^{-1} wet weight ~~(w.w.)~~ of tissue.

121 *2.3. Metal analysis*

122 Samples of 0.1-0.5 g wet weight of liver and muscle were digested in

123 concentrated nitric acid 65% (Baker~~),~~) at room temperature overnight, and were

124 heated at 80°C for 2 h. In order to determine the trace amounts of metal

125 contained in the feed, samples of commercial pellets were also digested (n=3).

126 Within each digestion series, appropriate blanks with ultra-pure water were also

127 subjected to the same procedure in order to ~~account for~~take background

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128 contamination levels into acount. After cooling, solutions were transferred to a

129 standard volume with ultra-pure water. Determination of metals (Cd, Cu, Zn, Fe,

130 Pb, Hg and Mn) was undertaken using an ICP-Mass (Elan DRC-I, Perkin-Elmer

131 Sciex). Samples of similar weight of certified reference material (DOLT-3 and

132 LUTS-1, National Research Council of Canada, Ottawa), were digested and

133 analyzed during each analytical run. The values of all elements found were in

134 good agreement with the certified values, with the recoveries ranging from 91%

135 to 104%.

136 *2.4. Data analysis and calculations*

137 Metal content ratio between muscle and liver was calculated in order to detect

138 changes in the distribution of metals as a consequence of the thermal

139 experimental conditions, and to showreveal the proportion of accumulated metal

140 in each tissue.

141 Bioaccumulation factor (BAF) was calculated for each metal-tissue-temperature

142 combination in order to detect the effects of temperature on the global

143 toxicokinetics among metals. ItThis was calculated as a quotient between the

144 metal concentration in each tissue and the metal content in the commercial

145 pellets as described by Dabrowska et al. (1996).

146 *2.5. Statistical analysis*

147 Statistical analyses were carried out using the software Stata 10 (Stata Corp).

148 Transformations of the data were performed when the assumption of normality

149 of residuals werewas not met. One-way ANOVA was used in the analysis of

150 data to check the influence of temperature inon each variable. The Scheffe test

7

151 was used as ~~a~~ post hoc test to ~~prove~~demonstrate the differences among the

152 three temperature groups. Two-way ANOVA, with temperature and metal as

153 fixed factors, was used in the statistical analysis of BAFs, followed by a post

154 hoc Bonferroni test. The Kruskal Wallis test was used when transformed data

155 did not present homoscedasticity. Pearson correlation coefficients (r) were

156 calculated between MT levels and metal content in liver, in order to measure the

157 strength of association between these variables. Results are presented as

158 means $\pm$ SEM and a p value lower than 0.05 was considered as statistically

159 significant.

160 **3. Results**

161 *3.1. Metal content in food and biometry*

162 Metal contents of food pellets were 0.~~378~~38 $\pm$ 0.05µg g^{-1} w.w. for Cd, 11.~~63~~6 $\pm$

163 0.~~74~~7 µg g^{-1} w.w. for Cu, ~~172.64 ± 8.89~~173 ± 9 µg g^{-1} w.w. for Fe, 40.~~14 ±~~

164 ~~2.78~~± 3 µg g^{-1} w.w. for Mn, 0.~~052~~05± 0.01µg g^{-1} w.w. for Hg, 0.15 $\pm$ 0.09 µg g^{-1}

165 w.w. for Pb and ~~138.94 ± 8.76~~139 ± 9 µg g^{-1} w.w. for Zn.

166 The weight and length ~~as well as~~together with the condition index calculated for

167 gilthead sea bream exposed to three experimental temperatures for three

168 months are shown in Table 1. As can be seen, temperature has no effect on the

169 condition index ~~and~~or weight~~, in~~. In contrast, fish kept at 30°C (p<0.05) ~~was~~were

170 found to be significantly shorter than those kept at 22°C and 27°C.

171 Insert Table 1

172 *3.2. Temperature effect on ~~metals content~~metal contents and their distribution*

173 *~~between~~among tissues*

8

174 Metal content ~~achieved~~reached in liver and muscle of gilthead sea bream is

175 shown in Figure 1. The ANOVA test shows a significant effect of temperature on

176 Cd, Hg and Pb content (p<0.001), and on concentration of the essential metals

177 Cu (p<0.01), Zn and Fe (p<0.001) in liver, but not on Mn content. Moreover, the

178 results show a significant effect of temperature on Mn, Fe and Zn content

179 (p<0.01) in muscle.

180 The muscle/liver ratios for each metal and temperature were calculated when

181 possible and are shown in Table 2. They indicate the distribution of metals after

182 temperature exposure and the proportion of each ~~one~~ in muscle in relation to

183 liver. The highest muscle/liver ratio for fish maintained at 22 and 27 ⁰C was

184 reached by Hg~~; however~~, whereas at 30 ⁰C the highest ratio was reached by

185 Mn. As can be seen in Table 2, calculated ratios for Hg and Fe decreased

186 significantly when temperature rose. Notably, and unlike for the other metals,

187 Mn ratio rose significantly with increasing temperature.

188 Insert Figure 1

189 Insert Table 2

190 *3.3. Temperature effect on toxicokinetic related processes: Bioaccumulation*

191 *Factor and MT induction*

192 The BAF values obtained for each metal-tissue-temperature combination are

193 shown in Table 3. The two-way ANOVA test shows significant differences

194 among between metals in muscle (p<0.001) at all the ~~temperature~~temperatures

195 tested. Hg BAF in this tissue is considerably higher in comparison with that

196 calculated for the other measured metals, reaching a value of 0.45 at 30⁰C. This

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means that muscle presents Hg content of almost a half of the feed content. In contrast, Mn BAF ~~exhibit~~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 27ºC and 30ºC, and reached the highest BAF registered in this study (3.20 at 30ºC). It should be noted that Hg ~~BAF~~BAFs in liver at 27ºC and 30ºC are, respectively, nearly 3 and 5 fold higher than those determined at 22ºC ~~respectively~~. A similar pattern to Hg was found for Cd, and in this case BAFs are 3 and 4 fold higher at 27ºC and 30ºC, respectively, than those determined at 22ºC. ~~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~~An 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

221 was reported in *Oncorhynchus kisutch,* as a consequence of the energetic

222 deficit after an elevated metabolism (Bowen et al., 2006).

223 Metal content of food pellets was in the same range as most of the values

224 reported in the literature for commercial pellets (Ciardullo et al.*,* 2008; Dang and

225 Wang, 2009; Mackee et al., 2008; Minganti et al., 2010). Cd, Pb and Hg feed

226 content are also below the established maximum permitted level in fish feed

227 according to ~~the~~ EU Directive 2002/32/EC and 2005/87/EC amending Annex I

228 to Directive 2002/32/EC.

229

230 *4.2. Effect of temperature on metal content and its distribution ~~between~~among*

231 *tissues*

232 Metal contents in tissues (Figure 1) were in the same range as most of the

233 values reported in the literature for farmed gilthead sea bream (Cretì et al.,

234 2010; Minghetti et al., 2008). It should be noted that Cd, Pb and Hg

235 concentrations measured in muscle of *Sparus aurata* in the present work are

236 below the established maximum permitted level in muscle of several species

237 according to ~~the~~ EU Regulation (EC) No 1881/2006. We have found a general

238 trend of increasing metal content in gilthead sea bream tissues with increasing

239 temperatures. The positive relationship between temperature and increased

240 metal uptake in different aquatic organisms~~,~~ has been explained by accelerated

241 biochemical and physiological processes, changes in the membrane

242 permeability, ~~and~~ alterations of metal transport systems, and binding to several

243 ligands (Baykan et al., 2007, Chowdhury and Blust, 2001; Jezierska and

244 Witeska, 2006; Lemus and Chung, 1999).

11

The significant increase in Hg content in liver at both~~,~~ 27ºC and 30ºC

~~comparing~~compared to ~~at~~ 22 ºC~~,~~ 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

~~in~~to 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 30ºC 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

12

270 ~~a~~an Mn receptor tissue as temperature increased. However, little information is

271 available concerning the effect of temperature on Mn accumulation in fish

272 muscle. Adam et al. (1997) described two groups of organs with different

273 elimination kinetics of ^{54}Mn, the first was the penetration or transit group, and

274 the second the receptor or storage organs. According to this, liver was classified

275 as ~~a~~an Mn distribution and elimination tissue, and muscle as a receptor ~~one~~.

276 The high levels of Cu in liver ~~from~~of fish kept at 30°C in comparison to those

277 kept at lower temperatures are in agreement with findings reported in several

278 studies. For instance, *Petenia kraussii* juveniles exposed to 22°C had lower Cu

279 basal concentrations than those exposed to 30°C (Lemus and Chung, 1999).

280 Temperature-related increase in Cu content has been explained by other

281 authors as a consequence of a different extent in the rising of accumulation and

282 elimination rates, resulting in a final positive balance for uptake (Glover et al.,

283 2003; Lemus and Chung, 1999).

284 In concordance with our results on Zn accumulation, Karakoç and Dinçer (2003)

285 reported a temperature induced accumulation of Zn in liver, and Van

286 Campenhout et al. (2007) described a promotion of Zn assimilation by

287 temperature in fish. ~~Also,~~ Zn uptake in the apical cell membrane of rainbow trout

288 intestine was also found to be a temperature-dependent process (Glover et al.,

289 2003). In addition to the above findings, and taking into account the

290 enhancement of Zn in muscle and liver of fish maintained at 30ºC, and that no

291 significant variation in the muscle/liver ratios was found in our study, we can

292 suggest that a temperature-induced disturbance of Zn homeostasis in *Sparus*

293 *aurata* may be occurring.

294 Levels of Fe in muscle and liver remained ~~quite~~fairly constant when temperature

295 increased from 22ºC to 27ºC, as can be expected for a tightly regulated metal

296 under physiological conditions. The sharp increase in Fe content in both

297 tissues, and the different ratio value found at 30°C may be due to Fe

298 homeostasis disturbance, indicating that some pathology is occurring. It should

299 be born in mind that Fe loading leads to free radical damage by the Fenton

300 reaction. Evidence in this direction has been found in fish and rats, suggesting a

301 role for Fe in hepatic injury following hyperthermia. (Bloomer et al., 2008;

302 Bowen et al., 2006; Skibba and Gwartney, 1997).

303 ~~Summarizing~~In summary, essential metals in muscle are strongly regulated

304 under physiological conditions, ~~however,~~although at 30ºC, their homeostasis is

305 disturbed as a result of a warmer temperature. The inverse relation

306 ~~among~~between muscle/liver ratio and temperature is a general trend for all the

307 metals studied, except Mn, which indicates that warmer temperatures facilitate

308 metal regulation processes, in such a way that there is a preferential

309 accumulation in liver thus protecting peripheral tissues.

310 *4.3. Temperature effect on toxicokinetic related processes: Bioaccumulation*

311 *Factor and MT induction*

312 BAF, the quotient between the metal concentration in tissue and the metal

313 content in the feed, allowed us to quantify the accumulation of metals from feed

314 in *Sparus aurata*, and to compare the temperature effect on bioaccumulation

315 among the determined metals.

316 Metabolic rates of ectothermic organisms may be strongly dependent on

317 temperature, and the same can be said for heavy ~~metal~~metals complexing with

318 cellular constituents, which may affect tissue distribution and BAF. We can

14

319 suggest that variations in toxicokinetics caused by temperature exist in some of

320 the studied ~~metal~~metals and tissues, although we cannot determine ~~if~~whether

321 these changes are a consequence of the temperature effect on assimilation

322 efficiency, or on feeding rate.

323 Hg presents the highest BAF values among metals for both tissues, at all

324 temperatures tested (Table 3). Hg accumulation depends on its speciation,

325 mainly because methylated Hg (MeHg) is better absorbed from the

326 gastrointestinal tract than the inorganic form in such a way that in mammals

327 95% is absorbed (Liu et al., 2008~~). Furthermore,~~), and furthermore it undergoes

328 extensive enterohepatic recycling (Liu et al., 2008). Taking ~~in~~into account all the

329 above, together with the fact that MeHg is the predominant form of Hg in fish

330 feed (>80%) (Amlund et al., 2007~~)~~), it is easy to understand the BAF values

331 found for Hg.

332 In agreement with hepatic Cd content, BAF values found in liver are higher at

333 warmer temperatures and, as discussed above, this may be due to the function

334 that this organ has in storage and detoxification of Cd (Jezierska and Witeska,

335 2006; Köck et al., 1996; Yang and Chen, 1996).

336 BAF for Pb in liver is lower than the BAF for the other two toxic metals (Cd and

337 Hg), which ~~could~~might be due to Pb preferential deposition into internal soft

338 tissues other than liver as reported in *Oncorhynchus mykiss* (Alves et al., 2006).

339 Mn shares a large number of transport mechanisms with other metals, ~~like~~ such

340 as transferrin, or the promiscuous divalent metal transporter (DMT) (Bury and

341 Grosell, 2003), and excretion mechanisms such as bile (Papavasiliou et al.,

342 1966), and for this reason, similar Mn behavior to other metals should be

343 expected. However, in this study Mn has a completely different reaction and

presents the lowest uptake from dietary sources, as the BAF values

suggest~~show~~. This may mean that Mn ~~stores~~is stored in other tissues than liver,

and this phenomenon may be amplified with increasing temperature.

It is important to note that ~~BAF~~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 ~~several~~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 ~~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

369 temperature, but in this case the reproductive state may also be involved (Gorbi

370 et al., 2005; Köck et al., 1996; Olsson et al., 1996; Rotchell et al., 2001). There

371 are only a few studies specially designed for elucidating the effect of

372 temperature on MT synthesis in fish. Van Cleef-Toedt et al. (2001)

373 demonstrated that non-spawning *Fundulus heteroclitus* exposed to thermal

374 stress exhibited significantly elevated liver, gill, and intestine MT mRNA

375 expression compared with controls.

376 In this study we have found an unusual correlation between MT and Fe content

377 in liver of *Sparus aurata*. MT is known to be a protein involved in protection

378 against oxidative stress (Viarengo et al 1999; Andrews, 2000; Gourgou et al.

379 2010), including heat-induced oxidative stress (Ivanina et al., 2009). On the

380 other hand, Fe may play a role in hepatic injury after hyperthermia and is

381 considered as an indicator of liver damage in mammals (Bloomer et al., 2008;

382 Bowen et al., 2006; Skibba and Gwartney, 1997). Fe excess is believed to

383 generate oxidative stress, understood as an increase in the steady state

384 concentration of oxygen radical intermediates (Puntarulo, 2005). Taking all the

385 above together we can suggest that the MT-Fe correlation may be due to the

386 accumulation of Fe in liver produced by hyperthermia, which can cause

387 oxidative stress which, in turn, produces MT induction .

388 **5. Conclusion**

389 The results obtained in this study show that metal bioaccumulation from non-

390 ~~experimental~~experimentally contaminated commercial feed and MT induction

391 takes place at warm temperatures in *Sparus aurata*. Furthermore, different

392 patterns of metal distribution among tissues and temperature indicate disturbed

393   essential metal homeostasis and higher levels of toxic metal bioaccumulation,

394   particularly of Hg and Cd. The interactions among water temperature, metal

395   uptake from feed and their distribution between tissues in these species exist,

396   but we can still we cannotnot provide a full mechanistic explanation for itthis.

397   Future research on the identification of the temperature-dependent mechanisms

398   involved in metal toxicokinetics is required in order to reach a better

399   understanding of temperature role in ecotoxicology and its implications in

400   aquaculture in a warming world context.

401   **Acknowledgments**

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562

Highlights

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- 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.

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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

highest bioaccumulation factor, and essential metal homeostasis was disturbed

in both tissues at warm temperatures. An enhancement of hepatic

metallothionein induction was found in fish exposed to the highest temperature.

Keywords: metal; metallothionein; bioaccumulation factor; temperature; *Sparus*

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

54 factors (Bowen et al., 2006; Chowdhury and Blust, 2001; Jezierska and

55 Witeska, 2006; Karakoç and Dinçer, 2003; Lemus and Chung, 1999).

56 Due to the growth in the activity of aquaculture in recent decades, it has

57 become of special relevance to learn the influence of the increase in

58 temperature on the possible routes of absorption, accumulation and elimination

59 of metals in these organisms in the context of global warming. Temperature

60 may be a determining factor in the capture, transporting and metabolism of the

61 metals incorporated through the feed, both of the essential metals which may

62 become toxic at high concentrations in the tissues, and of the non-essential

63 metals.

64 Metallothionein (MT) is a low-molecular-weight metal binding protein and is

65 known to play an important role in protection against heavy metal toxicity. In

66 addition to the detoxification of toxic metals such as Cd and Hg, MT is involved

67 in the maintenance of homeostasis of essential trace elements such as zinc

68 (Zn) and copper (Cu) (Coyle et al., 2002; Hamilton and Mehrle, 1986). Its role in

69 the protection against xenobiotics or in the cellular protection against oxidative

70 stress should be underlined (Coyle et al., 2002; Van Cleef-Toedt et al., 2001).

71 Although its synthesis is related to the metal exposure, its levels can be affected

72 by endogenous and exogenous factors such as the reproductive cycle or the

73 temperature (Van Cleef-Toedt et al., 2001). Variations in the water temperature

74 could directly or indirectly modify the behavior of this protein as regards the

75 bioaccumulation of metals, as well as its participation in toxicokinetic processes

76 (Baykan et al., 2007; Gorbi et al., 2005; Rotchell et al., 2001).

77 Given the lack of information as regards the effect of temperature on the

78 bioaccumulation of metals via dietary sources, and on the synthesis of MT, an

79   understanding of these processes in the light of the problem of global warming

80   and its repercussions on species of commercial interest such as *Sparus aurata*

81   is required. As such, the aims of this work are:

82   1) To determine the effect of temperature on the bioaccumulation of essential

83   (Cu, Fe, Mn and Zn) and non-essential (Cd, Hg and Pb) metals experimentally

84   via a non-contaminated commercial feed.

85   2) To discover whether temperature has any influence on the homeostasis of

86   the essential metals.

87   3) To elucidate the role of metallothionein in the above mentioned temperature

88   induced changes.

89   **2. Materials and Methods**

90   *2.1. Animal collection and maintenance*

91   Adult *Sparus aurata* were distributed and acclimated in 500 L tanks containing

92   seawater (37‰) at a constant temperature of 22°C, with continuous aeration

93   and natural photoperiod in a closed circuit for two months prior to the

94   experiment. Subsequently, the temperature of two of the experimental groups

95   was gradually increased to reach 27°C and 30°C respectively. Control groups of

96   fish were maintained at 22 ºC throughout the experiments. The animals were

97   fed with commercial pellets (1.5% of body mass per day) and the survival

98   percentage was 99%. Fish were kept under constant conditions for three

99   months. After this period, 6-7 fish were removed at random from each

100   experimental group and placed in water containing 30 mg L^{-1} of anesthetic clove

101   oil. Lengths and weights of gilthead sea bream were recorded. Fish were

102   sacrificed; livers and a piece of dorsal muscle tissue were dissected and

103   immediately frozen in liquid nitrogen and stored at -80 °C.

2.2. MT determination by differential pulse polarography

105 Approximately 0.2 g wet weight portions of frozen liver were homogenized using

106 ultra-turrax in 20 mM Tris-HCl buffer, 1 mM DTT and 0.2 mM PMSF pH 8.6 in

107 an ice bath. The homogenates were centrifuged at 30 000 *g* for 45 min at 4°C.

108 The supernatant was heated at 80°C for 10 min in order to denature high

109 molecular weight proteins and subsequently centrifuged at 30 000 g for 45 min

110 at 4°C,The heat-treated supernatant, containing thermally stable MT, was

111 separated from precipitated proteins. MT was measured using differential pulse

112 polarography (Ureña et al., 2007).

113 An aliquot of the heat-treated supernatant was added to the polarographic cell,

114 containing 20 mL hexammincobalt chloride buffer (the supporting electrolyte),

115 together with Triton-X (0.025% v/v). The cell was purged for 2 min with purified

116 N_2 prior to analysis. The polarographic response was measured during a

117 potential scan between -1.38 V and -1.7 V (Model 757 VA Computrace

118 Analyser, Methrom, Switzerland) in SMDE mode. Quantification of MT was

119 performed by using the standard addition method with rabbit liver MT I+II

120 (Sigma). Results are expressed as µg g^{-1} wet weight of tissue.

121 *2.3. Metal analysis*

122 Samples of 0.1-0.5 g wet weight of liver and muscle were digested in

123 concentrated nitric acid 65% (Baker) at room temperature overnight, and were

124 heated at 80°C for 2 h. In order to determine the trace amounts of metal

125 contained in the feed, samples of commercial pellets were also digested (n=3).

126 Within each digestion series, appropriate blanks with ultra-pure water were also

127 subjected to the same procedure in order to take background contamination

128 levels into acount. After cooling, solutions were transferred to a standard

129 volume with ultra-pure water. Determination of metals (Cd, Cu, Zn, Fe, Pb, Hg

130 and Mn) was undertaken using an ICP-Mass (Elan DRC-I, Perkin-Elmer Sciex).

131 Samples of similar weight of certified reference material (DOLT-3 and LUTS-1,

132 National Research Council of Canada, Ottawa), were digested and analyzed

133 during each analytical run. The values of all elements found were in good

134 agreement with the certified values, with the recoveries ranging from 91% to

135 104%.

136 *2.4. Data analysis and calculations*

137 Metal content ratio between muscle and liver was calculated in order to detect

138 changes in the distribution of metals as a consequence of the thermal

139 experimental conditions, and to reveal the proportion of accumulated metal in

140 each tissue.

141 Bioaccumulation factor (BAF) was calculated for each metal-tissue-temperature

142 combination in order to detect the effects of temperature on the global

143 toxicokinetics among metals. This was calculated as a quotient between the

144 metal concentration in each tissue and the metal content in the commercial

145 pellets as described by Dabrowska et al. (1996).

146 *2.5. Statistical analysis*

147 Statistical analyses were carried out using the software Stata 10 (Stata Corp).

148 Transformations of the data were performed when the assumption of normality

149 of residuals was not met. One-way ANOVA was used in the analysis of data to

150 check the influence of temperature on each variable. The Scheffe test was used

151   as a post hoc test to demonstrate the differences among the three temperature

152   groups. Two-way ANOVA, with temperature and metal as fixed factors, was

153   used in the statistical analysis of BAFs, followed by a post hoc Bonferroni test.

154   The Kruskal Wallis test was used when transformed data did not present

155   homoscedasticity. Pearson correlation coefficients (r) were calculated between

156   MT levels and metal content in liver, in order to measure the strength of

157   association between these variables. Results are presented as means $\pm$ SEM

158   and a p value lower than 0.05 was considered as statistically significant.

159   **3. Results**

160   *3.1. Metal content in food and biometry*

161   Metal contents of food pellets were $0.38 \pm 0.05 \mu g\ g^{-1}$ w.w. for Cd, $11.6 \pm 0.7\ \mu g$

162   g^{-1} w.w. for Cu, $173 \pm 9\ \mu g\ g^{-1}$ w.w. for Fe, $40. \pm 3\ \mu g\ g^{-1}$ w.w. for Mn, $0.05\pm$

163    $0.01 \mu g\ g^{-1}$ w.w. for Hg, $0.15 \pm 0.09\ \mu g\ g^{-1}$ w.w. for Pb and $139 \pm 9\ \mu g\ g^{-1}$ w.w.

164   for Zn.

165   The weight and length together with the condition index calculated for gilthead

166   sea bream exposed to three experimental temperatures for three months are

167   shown in Table 1. As can be seen, temperature has no effect on the condition

168   index or weight. In contrast, fish kept at 30°C ($p<0.05$) were found to be

169   significantly shorter than those kept at 22°C and 27°C.

170   Insert Table 1

171   *3.2. Temperature effect on metal contents and their distribution among tissues*

172   Metal content reached in liver and muscle of gilthead sea bream is shown in

173   Figure 1. The ANOVA test shows a significant effect of temperature on Cd, Hg

174 and Pb content (p<0.001), and on concentration of the essential metals Cu

175 (p<0.01), Zn and Fe (p<0.001) in liver, but not on Mn content. Moreover, the

176 results show a significant effect of temperature on Mn, Fe and Zn content

177 (p<0.01) in muscle.

178 The muscle/liver ratios for each metal and temperature were calculated when

179 possible and are shown in Table 2. They indicate the distribution of metals after

180 temperature exposure and the proportion of each in muscle in relation to liver.

181 The highest muscle/liver ratio for fish maintained at 22 and 27 ºC was reached

182 by Hg, whereas at 30 ºC the highest ratio was reached by Mn. As can be seen

183 in Table 2, calculated ratios for Hg and Fe decreased significantly when

184 temperature rose. Notably, and unlike for the other metals, Mn ratio rose

185 significantly with increasing temperature.

186 Insert Figure 1

187 Insert Table 2

188 *3.3. Temperature effect on toxicokinetic related processes: Bioaccumulation*

189 *Factor and MT induction*

190 The BAF values obtained for each metal-tissue-temperature combination are

191 shown in Table 3. The two-way ANOVA test shows significant differences

192 among between metals in muscle (p<0.001) at all the temperatures tested. Hg

193 BAF in this tissue is considerably higher in comparison with that calculated for

194 the other measured metals, reaching a value of 0.45 at 30ºC. This means that

195 muscle presents Hg content of almost a half of the feed content. In contrast, Mn

196 BAF exhibits the lowest BAF for all temperature exposures.

197 Statistical analysis of BAFs also showed significant differences among metals in

198 liver (p<0.001). BAF for Hg in liver differed from the BAFs for other metals at

199 27ºC and 30ºC, and reached the highest BAF registered in this study (3.20 at

200 30ºC). It should be noted that Hg BAFs in liver at 27ºC and 30ºC are,

201 respectively, nearly 3 and 5 fold higher than those determined at 22ºC. A similar

202 pattern to Hg was found for Cd, and in this case BAFs are 3 and 4 fold higher at

203 27ºC and 30ºC, respectively, than those determined at 22ºC. Mn BAFs in liver

204 are the lowest among the studied metals.

205 Insert Table 3

206 MT levels determined in liver for the three experimental temperature groups are

207 shown in Figure 1. As can be seen, there is a significant thermal effect on

208 hepatic MT content. An MT significant induction is found in fish exposed to the

209 highest temperature (p<0.001). Furthermore, significant positive correlations

210 between MT and Cd in liver (r= 0.52; p<0.05), Hg (r= 0.78; p<0.001), Zn (r=

211 0.82; p<0.001), Cu (r= 0.82; p<0.001) and Fe (r= 0.78; p<0.001) have been

212 found, but not with Pb and Mn.

213 **4. Discussion**

214 *4.1. Metal content in food and biometry*

215 Length differed significantly between animals exposed to 22°C and 30°C (Table

216 1), which could be due to an increase in energetic demand related to an

217 increase in metabolic rate. A reduced growth and length at high temperature

218 was reported in *Oncorhynchus kisutch,* as a consequence of the energetic

219 deficit after an elevated metabolism (Bowen et al., 2006).

220 Metal content of food pellets was in the same range as most of the values

221 reported in the literature for commercial pellets (Ciardullo et al.*,* 2008; Dang and

222 Wang, 2009; Mackee et al., 2008; Minganti et al., 2010). Cd, Pb and Hg feed

223 content are also below the established maximum permitted level in fish feed

224 according to EU Directive 2002/32/EC and 2005/87/EC amending Annex I to

225 Directive 2002/32/EC.

226

227 *4.2. Effect of temperature on metal content and its distribution among tissues*

228 Metal contents in tissues (Figure 1) were in the same range as most of the

229 values reported in the literature for farmed gilthead sea bream (Cretì et al.,

230 2010; Minghetti et al., 2008). It should be noted that Cd, Pb and Hg

231 concentrations measured in muscle of *Sparus aurata* in the present work are

232 below the established maximum permitted level in muscle of several species

233 according to EU Regulation (EC) No 1881/2006. We have found a general trend

234 of increasing metal content in gilthead sea bream tissues with increasing

235 temperatures. The positive relationship between temperature and increased

236 metal uptake in different aquatic organisms has been explained by accelerated

237 biochemical and physiological processes, changes in the membrane

238 permeability, alterations of metal transport systems, and binding to several

239 ligands (Baykan et al., 2007, Chowdhury and Blust, 2001; Jezierska and

240 Witeska, 2006; Lemus and Chung, 1999).

241 The significant increase in Hg content in liver at both 27ºC and 30ºC compared

242 to at 22 ºC indicates strong temperature dependence. Additionally, the

243 muscle/liver ratio (Table 2) confirms the preferential affinity of Hg to lipidic

244   tissues such as the liver. Using an experimental trophic chain, Boudou et al.

245   (1979) demonstrated that raising the temperature of the environment has a

246   synergic effect on the quantities of Hg bioaccumulated by a carnivorous fish.

247   Cd content in liver also increased with rising temperature. Liver, as is well

248   known, is an organ involved in storage and detoxification of Cd, and a

249   progressive accumulation of dietary Cd over time has been explained by the

250   continuous transference via the portal system from the digestive tract to the liver

251   (Handy, 1993). In previous temperature effect studies in fish, the metal was

252   supplied as waterborne Cd (Douben, 1989; Köck et al., 1996) and the

253   temperature-dependent increase of metal content was explained by a greater

254   increase in uptake rate than the increase found in the elimination rate (Douben,

255   1989), although mechanisms involved in Cd accumulation may differ to a great

256   extent depending on the Cd exposure source.

257   The highest Pb content in liver was reached by fish kept at 27°C. In some

258   studies Pb uptake rate has been affirmed to be temperature-dependent in fish

259   (Köck et al., 1996).

260   An enhancement of Mn in muscle of fish maintained at 30ºC in relation to those

261   kept at colder temperatures was determined, nevertheless no significant

262   variation in Mn was found in liver. However, when muscle/liver ratios were

263   calculated, a significant effect of the temperature was detected in such a way

264   that the ratio was higher at the warmer temperature, with the muscle acting as

265   an Mn receptor tissue as temperature increased. However, little information is

266   available concerning the effect of temperature on Mn accumulation in fish

267   muscle. Adam et al. (1997) described two groups of organs with different

268   elimination kinetics of ^{54}Mn, the first was the penetration or transit group, and

269 the second the receptor or storage organs. According to this, liver was classified

270 as an Mn distribution and elimination tissue, and muscle as a receptor.

271 The high levels of Cu in liver of fish kept at 30°C in comparison to those kept at

272 lower temperatures are in agreement with findings reported in several studies.

273 For instance, *Petenia kraussii* juveniles exposed to 22°C had lower Cu basal

274 concentrations than those exposed to 30°C (Lemus and Chung, 1999).

275 Temperature-related increase in Cu content has been explained by other

276 authors as a consequence of a different extent in the rising of accumulation and

277 elimination rates, resulting in a final positive balance for uptake (Glover et al.,

278 2003; Lemus and Chung, 1999).

279 In concordance with our results on Zn accumulation, Karakoç and Dinçer (2003)

280 reported a temperature induced accumulation of Zn in liver, and Van

281 Campenhout et al. (2007) described a promotion of Zn assimilation by

282 temperature in fish. Zn uptake in the apical cell membrane of rainbow trout

283 intestine was also found to be a temperature-dependent process (Glover et al.,

284 2003). In addition to the above findings, and taking into account the

285 enhancement of Zn in muscle and liver of fish maintained at 30ºC, and that no

286 significant variation in the muscle/liver ratios was found in our study, we can

287 suggest that a temperature-induced disturbance of Zn homeostasis in *Sparus*

288 *aurata* may be occurring.

289 Levels of Fe in muscle and liver remained fairly constant when temperature

290 increased from 22ºC to 27ºC, as can be expected for a tightly regulated metal

291 under physiological conditions. The sharp increase in Fe content in both

292 tissues, and the different ratio value found at 30°C may be due to Fe

293 homeostasis disturbance, indicating that some pathology is occurring. It should

294　be born in mind that Fe loading leads to free radical damage by the Fenton

295　reaction. Evidence in this direction has been found in fish and rats, suggesting a

296　role for Fe in hepatic injury following hyperthermia. (Bloomer et al., 2008;

297　Bowen et al., 2006; Skibba and Gwartney, 1997).

298　In summary, essential metals in muscle are strongly regulated under

299　physiological conditions, although at 30ºC their homeostasis is disturbed as a

300　result of a warmer temperature. The inverse relation between muscle/liver ratio

301　and temperature is a general trend for all the metals studied except Mn, which

302　indicates that warmer temperatures facilitate metal regulation processes in such

303　a way that there is a preferential accumulation in liver thus protecting peripheral

304　tissues.

305　*4.3. Temperature effect on toxicokinetic related processes: Bioaccumulation*

306　*Factor and MT induction*

307　BAF, the quotient between the metal concentration in tissue and the metal

308　content in the feed, allowed us to quantify the accumulation of metals from feed

309　in *Sparus aurata*, and to compare the temperature effect on bioaccumulation

310　among the determined metals.

311　Metabolic rates of ectothermic organisms may be strongly dependent on

312　temperature, and the same can be said for heavy metals complexing with

313　cellular constituents, which may affect tissue distribution and BAF. We can

314　suggest that variations in toxicokinetics caused by temperature exist in some of

315　the studied metals and tissues, although we cannot determine whether these

316　changes are a consequence of the temperature effect on assimilation efficiency,

317　or on feeding rate.

318 Hg presents the highest BAF values among metals for both tissues at all

319 temperatures tested (Table 3). Hg accumulation depends on its speciation,

320 mainly because methylated Hg (MeHg) is better absorbed from the

321 gastrointestinal tract than the inorganic form in such a way that in mammals

322 95% is absorbed (Liu et al., 2008), and furthermore it undergoes extensive

323 enterohepatic recycling (Liu et al., 2008). Taking into account all the above,

324 together with the fact that MeHg is the predominant form of Hg in fish feed

325 (>80%) (Amlund et al., 2007), it is easy to understand the BAF values found for

326 Hg.

327 In agreement with hepatic Cd content, BAF values found in liver are higher at

328 warmer temperatures and, as discussed above, this may be due to the function

329 that this organ has in storage and detoxification of Cd (Jezierska and Witeska,

330 2006; Köck et al., 1996; Yang and Chen, 1996).

331 BAF for Pb in liver is lower than the BAF for the other two toxic metals (Cd and

332 Hg), which might be due to Pb preferential deposition into internal soft tissues

333 other than liver as reported in *Oncorhynchus mykiss* (Alves et al., 2006).

334 Mn shares a large number of transport mechanisms with other metals such as

335 transferrin, or the promiscuous divalent metal transporter (DMT) (Bury and

336 Grosell, 2003), and excretion mechanisms such as bile (Papavasiliou et al.,

337 1966), and for this reason similar Mn behavior to other metals should be

338 expected. However, in this study Mn has a completely different reaction and

339 presents the lowest uptake from dietary sources, as the BAF values show. This

340 may mean that Mn is stored in other tissues than liver, and this phenomenon

341 may be amplified with increasing temperature.

342 It is important to note that BAFs of Cu and Zn in liver are almost the same when

343 determined for the same temperature, which indicates that a similar fraction of

344 metal contained in feed is retained, and also that temperature has a similar

345 effect on the overall metabolism of both elements.

346 Essential metals have transport systems that include both specific and general

347 mechanisms. They are strongly regulated in organisms under non-stressful

348 conditions, and consequently environmental factor variations should not greatly

349 affect their concentration. On the other hand, toxic metals have no specific

350 mechanisms and use essential element transport proteins and channels, such

351 as divalent metal transporter (DMT1), diverse ZIP proteins or MT (Bury et al.,

352 2003; Coyle et al., 2002; He et al., 2006; Vesey et al., 2010). It is clear that the

353 common mechanisms may be affected by temperature to a different degree or

354 in a different way depending on the metal and tissue. For instance, the

355 temperature-dependent increase of membrane fluidity and physical diffusion

356 rate may be of special relevance for Hg accumulation (Foulkes, 2000). It is

357 difficult to determine at which level the mechanisms implicated in the

358 toxicokinetics of each metal are influenced by temperature.

359 The effect of temperature on MT level in liver could be a direct thermal

360 response, or may be related to the increase in metal content. MT synthesis is

361 considered one of the best-known biochemical detoxification mechanisms for

362 metal and it is widely demonstrated that its induction may be influenced by

363 metal contamination. It is very common to try to relate seasonal variability to

364 temperature, but in this case the reproductive state may also be involved (Gorbi

365 et al., 2005; Köck et al., 1996; Olsson et al., 1996; Rotchell et al., 2001). There

366 are only a few studies specially designed for elucidating the effect of

367 temperature on MT synthesis in fish. Van Cleef-Toedt et al. (2001)

368 demonstrated that non-spawning *Fundulus heteroclitus* exposed to thermal

369 stress exhibited significantly elevated liver, gill, and intestine MT mRNA

370 expression compared with controls.

371 In this study we have found an unusual correlation between MT and Fe content

372 in liver of *Sparus aurata*. MT is known to be a protein involved in protection

373 against oxidative stress (Viarengo et al 1999; Andrews, 2000; Gourgou et al.

374 2010), including heat-induced oxidative stress (Ivanina et al., 2009). On the

375 other hand, Fe may play a role in hepatic injury after hyperthermia and is

376 considered as an indicator of liver damage in mammals (Bloomer et al., 2008;

377 Bowen et al., 2006; Skibba and Gwartney, 1997). Fe excess is believed to

378 generate oxidative stress, understood as an increase in the steady state

379 concentration of oxygen radical intermediates (Puntarulo, 2005). Taking all the

380 above together we can suggest that the MT-Fe correlation may be due to the

381 accumulation of Fe in liver produced by hyperthermia, which can cause

382 oxidative stress which, in turn, produces MT induction .

383 **5. Conclusion**

384 The results obtained in this study show that metal bioaccumulation from non-

385 experimentally contaminated commercial feed and MT induction takes place at

386 warm temperatures in *Sparus aurata*. Furthermore, different patterns of metal

387 distribution among tissues and temperature indicate disturbed essential metal

388 homeostasis and higher levels of toxic metal bioaccumulation, particularly of Hg

389 and Cd. The interactions among water temperature, metal uptake from feed and

390 their distribution between tissues in these species exist, but we can still not

391 provide a full mechanistic explanation for this. Future research on the

392 identification of the temperature-dependent mechanisms involved in metal

393 toxicokinetics is required in order to reach a better understanding of

394 temperature role in ecotoxicology and its implications in aquaculture in a

395 warming world context.

396 **Acknowledgments**

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557

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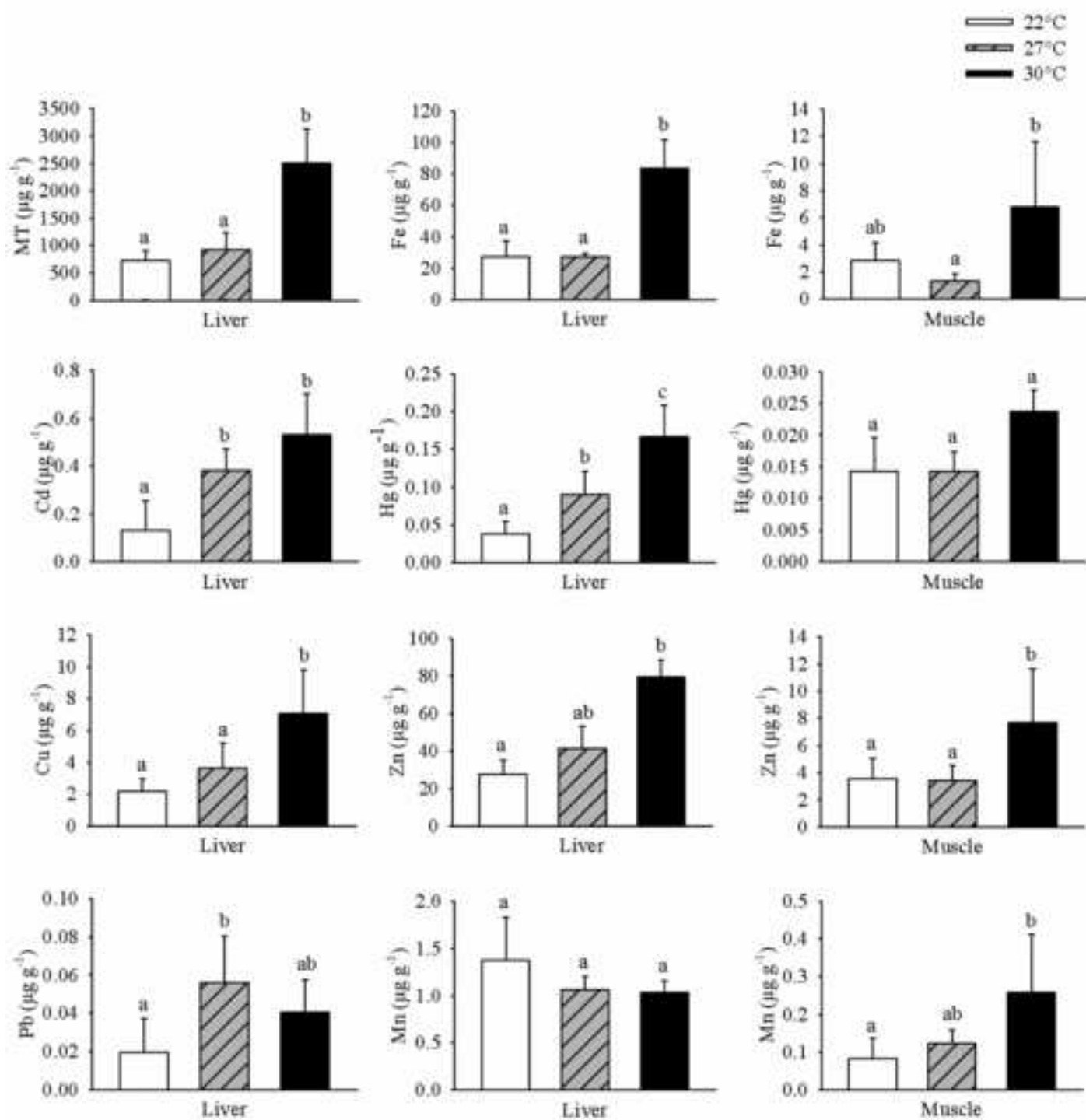
Table 1. Biometry of *Sparus aurata* kept under temperature exposure for three months. Data represent mean ± SEM (n≥6). Means that do not share the same letter differ significantly (p<0.05).

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

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

	22°C	27°C	30°C
Fe	0.09 ± 0.02 [a]	0.06 ± 0.01 [a]	0.07 ± 0.05 [b]
Mn	0.07 ± 0.04 [a]	0.11 ± 0.02 [ab]	0.25 ± 0.02 [b]
Hg	0.43 ± 0.23 [a]	0.16 ± 0.06 [b]	0.15 ± 0.09 [b]
Zn	0.14 ± 0.08 [a]	0.08 ± 0.04 [a]	0.10 ± 0.06 [a]

Table 3 corrected
[Click here to download Table: Table 3correct.doc](#)

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}