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1 Thermal imprinting modifies bone homeostasis in cold challenged sea bream (*Sparus*
2 *aurata,* L.)
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13 **Running title:** Thermal imprinting affects bone
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28 response, teleost fish.

Summary Statement

Variation in water temperature during early development in sea bream alters the response of adult fish to a cold challenge and is associated with a change in whole animal physiology and bone homeostasis.

66 ## Abstract

67 Fish are ectotherms and temperature plays a determinant role in their physiology, biology and
68 ecology and is a driver of seasonal responses. The present study assessed how thermal imprinting
69 during embryonic and larval stages modified the response of adult fish to low water temperature.
70 We targeted the gilthead sea bream that develops a condition known as winter syndrome when it
71 is exposed to low water temperatures. Eggs and larvae of sea bream were exposed to four different
72 thermal regimes and then the response of the resulting adults to a low temperature challenge was
73 assessed. Sea bream exposed to a high-low thermal regime as eggs and larvae (HLT, 22ºC until
74 hatch and then 18ºC until larvae-juvenile transition) had increased plasma cortisol and lower
75 sodium and potassium in response to a cold challenge compared to the other thermal history
76 groups. Plasma glucose and osmolality were increased in cold challenge HLT fish relative to the
77 unchallenged HLT fish. Cold challenge modified bone homeostasis/responsiveness in the low-
78 high thermal regime group (LHT) relative to other groups and *ocn*, *ogn1/2*, *igf1*, *gr* and *trα/β*
79 transcripts were all down-regulated. In the low temperature group (LT) and HLT group
80 challenged with a low temperature, ALP/TRAP activities were decreased relative to unchallenged
81 groups and bone calcium content also decreased in the LT group. Overall, the results indicate that
82 thermal imprinting during early development of sea bream causes a change in the physiological
83 response of adults to a cold challenge.

Introduction

Fish are ectotherms and their body temperature is in equilibrium with the external thermal conditions (Mozes et al., 2011). This means that changes in ambient water temperature directly affect the cell cycle, metabolism, membrane fluidity and at the molecular level influence transcription, translation, post-translational processing and protein structure (Somero, 2010). The overall effect of temperature is apparent as an overt change in whole animal physiology such as growth rate, feeding rate and body composition (Clarke and Johnston, 1999; Greene and Selivonchick, 1987; Wang et al., 1987; Wiegand et al., 1988). Temperature therefore plays a determinant role in fish physiology, biology and ecology and is a driver of seasonal responses (Mozes et al., 2011; Somero, 2005). Gilthead sea bream (*Sparus aurata* L.) is eurythermal and in the wild is exposed to a broad range of ambient water temperatures (11ºC to 26ºC) and behavioral thermoregulation allows them to avoid temperature extremes (Davis, 1988).

Aquaculture production of the gilthead sea bream is concentrated in the Mediterranean, from Turkey to Spain (FEAP, 2015) and since fish are caged they are unable to avoid seasonal fluctuations in water temperature (Tattersall et al., 2012; Tort et al., 2011). Under aquaculture conditions, a prolonged winter with water temperatures below 13ºC often leads to mortality of unknown aetiology in sea bream and not strongly associated with a specific pathogen (Padrós et al., 1996; Sarusic, 1999) that has been termed Winter Syndrome or Winter Disease (Tort et al., 2011). This syndrome is a multifactorial condition associated with a high but transient (24 to 48 hours) rise in plasmatic levels of cortisol and triggers a classical stress response with the associated secondary effects (Rotllant et al., 2000; Sala-Rabanal et al., 2003). A reduction in food intake (Rotllant et al., 2000; Tort et al., 2004) or starvation (Ibarz et al., 2007; Ibarz et al., 2005; Ibarz et al., 2003) occurs and fish affected by winter syndrome become lethargic. Immunocompetence is also severely depressed (Berthe et al., 1995; Doménech et al., 1997; Tort et al., 1998; Vargas-Chacoff et al., 2009), osmoregulatory capacity is impaired (Ibarz et al., 2010a) and histopathological changes occur in the liver, exocrine pancreas, digestive tract and muscle (Gallardo et al., 2003; Ibarz et al., 2010a; Ibarz et al., 2010b; Sala-Rabanal et al., 2003). Despite the efforts to understand how fish cope with winter syndrome and the mechanisms underlying this disease, no consideration has been given to how variation in water temperature during early ontogeny might modulate the response of fish to environmental stressors in adult life, specifically to a cold water challenge.

Bone plays an important role in plasma ion homeostasis, is intimately linked to muscle growth and is essential for load bearing and movement (Hall, 2005). Nonetheless, the impact of temperature on bone is largely unexplored, although evidence exists that low temperature causes metabolic changes (in plasma ions and starvation) that can influence bone homeostasis (Doherty et al., 2015; Takagi, 2001; Vieira et al., 2013). However, the impact of winter syndrome on bone homeostasis and potentially calcium balance and malformations is unstudied.

121 Recently considerable interest has been focused on determining the impact of thermal
122 imprinting during embryonic and larval stages on the phenotypic plasticity of adult fish in part as
123 a response to growing concern about the likely impact of global warming (Somero, 2005; Wood
124 and McDonald, 1997). Thermal imprinting in early stages has a persistent effect on gene
125 expression in subsequent stages (Garcia de la Serrana et al., 2012; Johnston et al., 2009; Jonsson
126 and Jonsson, 2014; Scott and Johnston, 2012) and gene methylation and non-coding RNA have
127 been suggested to contribute to the effect of temperature on developmental plasticity (Bizuayehu
128 et al., 2015; Campos et al., 2014). Evidence has been gathered revealing that the thermal regime
129 during early development can influence the juvenile stress response (Auperin and Geslin, 2008;
130 Varsamos et al., 2006), muscle growth (Alami-Durante et al., 2007; Galloway et al., 1999; Garcia
131 de la Serrana et al., 2012; Johnston et al., 2009; Macqueen et al., 2008; Steinbacher et al., 2011),
132 and the incidence and character of skeletal deformities (Boglione and Costa, 2011). However,
133 little is known about how embryonic or larval temperature regimes affect the ability of fish to
134 cope with temperature changes in adult life. It is known that thermal imprinting in zebrafish
135 (*Danio rerio)* embryos induce modified thermal tolerance in juveniles exposed to higher than
136 normal culture temperatures (Schaefer and Ryan, 2006), but the effect of temperature during
137 development on the physiological response to cold in adults is unstudied. In addition, the impact
138 of early life temperatures on bone homeostasis have not previously been studied in adult teleost
139 fish. Most of the studies that exist have looked at the effect of increased temperature on skeletal
140 development in species such as tilapia (Campinho et al., 2004), European sea bass *Dicentrarachus*
141 *labrax* (Koumoundouros et al., 2001), Atlantic salmon (Takle et al., 2005) and gilthead sea bream
142 (Boglione and Costa, 2011) or how it affects the incidence of malformations, an issue of
143 importance to aquaculture (Boglione et al., 2013; Koumoundouros, 2010).
144 Taking into consideration the role of temperature on thermal imprinting and subsequent
145 performance of juveniles and adults and the known vulnerability of the skeleton to temperature
146 induced changes in larvae (Divanach et al., 1996; Polo et al., 1991; Sfakianakis et al., 2011), we
147 hypothesized that early thermal history from embryogenesis through the larvae-juvenile transition
148 might influence the response of bone in adults to changes in water temperature characteristic of
149 winter. To test this hypothesis adult fish with different thermal histories were exposed to a cold
150 challenge typical of that experienced during winter. Since activation of the stress axis has
151 previously been reported in winter syndrome (Rotllant et al., 2000), we assessed the response of
152 adult fish with different thermal histories to a temperature drop by measuring plasma parameters
153 associated with the stress response. The impact of thermal imprinting on bone metabolism during
154 the temperature challenge was assessed by analysis of osteoblast and osteoclast activity by
155 measuring the enzymatic activity of alkaline phosphatase (ALP, Dimai et al., 1998) and tartrate
156 resistant acid phosphatase (TRAP, Persson et al., 1995), respectively, and determining the ash
157 and calcium content of bone and the abundance of transcripts associated with the bone matrix. To

158 assess if part of the effect of thermal imprinting occurred through modification of factors that
159 regulate bone responsiveness, we analyzed the relative gene expression of regulatory factors like
160 insulin-like growth factor 1 (*igf1*), glucocorticoid receptor (*gr*) and thyroid receptors in bone (*trα*
161 and *trβ*). Overall the objective of the study was to assess if thermal regime during sea bream
162 development could influence the physiological response of young adults to a cold water challenge.
163
164
165 ## Material and Methods

166 ### Early life programming

167 All the procedures of early life temperature treatments and stress challenge were
168 performed at the Institute for Aquaculture and Food Technology Research (IRTA), St. Carles de
169 la Ràpita, Spain, in a temperature-controlled seawater recirculation system (IRTAmar™). All
170 animal handling procedures were approved by the Ethics and Animal Care Committee (4998-
171 T9900002) and complied with the guidelines of the European Union Council (86/609/EU),
172 Spanish and Catalan Governments legislation.
173 Detailed information about the thermal imprinting experiments are provided in Garcia de
174 la Serrana et al. (2012). In brief, fertilized eggs of gilthead sea bream (fertilization rate = 92%)
175 were maintained at two different temperatures during embryogenesis, 18ºC (low temperature, LT)
176 or 22ºC (high temperature, HT) in two independent temperature-controlled seawater recirculation
177 systems. The two systems included two tanks of 2 m^3, and each contained two incubators (30 L)
178 containing 110 mL of fertilized eggs. At hatching, larvae from replicate incubators within each
179 temperature treatment were pooled, as no differences in hatching rate were observed, and they
180 were then subdivided to generate the four different temperature regimes (2 replicate tanks/group,
181 Fig. 1A). The temperature regimes were selected considering the two extreme temperatures of the
182 optimal range for early life development of gilthead sea bream (18 and 22ºC) (Hough, 2010;
183 Mozes et al., 2011): i) 18ºC from egg incubation through to hatching and up until larvae-juvenile
184 transition (low temperature, LT); ii) 22ºC from egg incubation through to hatching and up until
185 larvae-juvenile transition (high temperature, HT); iii) 18ºC from egg incubation up until hatching
186 and then 22ºC until larvae-juvenile transition (low-high temperature, LHT); iv) 22ºC from egg
187 incubation through to hatching and then 18ºC up until larvae-juvenile transition (high-low
188 temperature, HLT). All treatment groups of juvenile fish were then maintained for seven months
189 in duplicate 2 m^3 tanks per group, in a semi-closed recirculating sea water system with 5-10%
190 water renewal/week, under a constant water temperature regime (21-22ºC). Juvenile fish were fed
191 five times per day at 3% (kg/kg fish) with a commercial diet (OptiBream™).

192 A relatively large stock of thermally imprinted fish (adult fish in which the eggs and
193 larvae were reared under different temperature regimes, approx. 700-900 per thermal regime)
194 were generated and were used for several independent experiments (Garcia de la Serrana et al.
195 (2012); Mateus et al., in press). Fish used for the present cold challenge experiment were age
196 matched (7 months' post-hatch). Potential sex-related differences were not expected since the sea
197 bream is a hermaphrodite and during the first year mature as males (Pinto et al., 2006; Zohar et
198 al., 1978). However, significant differences in weight and length existed between fish from the
199 different thermal regimes (P<0.001; Table 1). The biometric differences detected in the present
200 study between thermally imprinted fish were confirmed in a subsequent stress challenge
201 experiment performed with 9 months post-hatch sea bream from the same stock of fish (Mateus
202 et al., in press).
203

204 **Cold challenge and sampling**

205 To assess if thermal imprinting could modify the physiological response of young adult
206 sea bream subjected to a cold water challenge, duplicate tanks of fish from each thermal regime
207 (LT, LHT, HT and HLT) were randomly divided into two groups: the water temperature of the
208 control groups was 23.0±1.0ºC and the cold challenge groups was 13.0±1.0ºC (Fig. 1B). Water
209 temperature was progressively reduced at a rate of 1ºC per day, until the target temperature, 13ºC,
210 was attained. Sea bream (n=10/group/ tank, see Table 1 for data on body weight, length, condition
211 factor [K] and hepatosomatic index [HSI]) were exposed to reduced water temperature for 15
212 days. The circuit consisted of 200 L fiberglass tanks in a semi-closed sea water system at pH 7.5-
213 8.0, 35-36‰ salinity and >80% oxygen saturation and maintained under a 12 h light/12 h dark
214 photoperiod. Fish were fed to satiation and this corresponded to approximately 3% body weight
215 daily using a commercial diet (OptiBream^{TM}) for the control groups and 1% body weight daily
216 for the cold challenge since they would not eat more due to the cold stress. Uneaten food was
217 siphoned daily from the bottom of the experimental tanks.
218 For sampling, fish were sacrificed with an overdose (450 ppm) of 2-phenoxyethanol
219 (Sigma-Aldrich, USA), blotted dry and blood collected from the caudal vein using a heparinized
220 syringe, centrifuged at 10,000 rpm for 4 minutes at 4ºC, and the plasma stored at -20ºC. Haemal
221 vertebrae (bone) were collected into RNA later for subsequent RNA extraction, enzymatic assays
222 and calcium and mineral content analysis. Vertebrae samples were incubated overnight at 4ºC in
223 RNA later and then stored at -80ºC until analyses.

224 **Plasma analyses**

225 Plasma cortisol (ng.mL^{-1}) was measured in duplicate using a validated radioimmunoassay
226 (RIA, Rotllant et al., 2005). Plasma osmolality (mmol.Kg^{-1}) was determined using a vapor
227 pressure osmometer (VaproWescor 5520, Utah, USA) and sodium (Na^+) and potassium (K^+)
228 concentrations were determined by flame photometry (BWB Technologies, USA) and the results
229 expressed in mM (n=10/group).

230 Plasma glucose (mmol.L^{-1}) and total calcium (Ca^{2+}, mmol.L^{-1}) were measured with
231 glucose oxidase-peroxidase and o-Cresolphtalein colourimetric assays, respectively (Spinreact
232 1001190 and 1001061, Spain). Total protein (mg.mL^{-1}) was measured in diluted plasma samples
233 (1:40) using a colorimetric assay (#500-0006, BioRad, USA) and a standard curve prepared using
234 bovine serum albumin (Quick Start BSA Standard Set, #500-0207, BioRad, USA). Analysis of
235 the colourimetric assays was performed using a micro plate reader (Benchmark, BioRad, USA)
236 set at the appropriate wavelength (510 nm for glucose, 570 nm for Ca^{2+} and 595 nm for protein).

237 **Bone TRAP and ALP activities**

238 TRAP and ALP activities were measured as described in Guerreiro et al. (2013). Samples
239 of frozen vertebrae (n=10/group) were crushed and then 8-12 mg used for each assay. Two-
240 hundred µL of 20 mM Tartrate in NaAc buffer (0.1 M, pH 5.3) was added to 8-12 mg of crushed
241 vertebra and used to determine the TRAP activity. To determine the ALP activity 200 µL of 0.1
242 M Tris–HCl (pH 9.5), 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$ buffer was added to 8-12 mg of crushed
243 vertebra. Each sample was assayed in duplicate and color was developed for 20 min at 24ºC before
244 addition of 200 µL of the substrate para-nitrophenyl phosphate (pNPP, 5 mM). The reactions were
245 stopped by adding 200 µL of 2 M NaOH and the absorbance was measured at 405 nm. A standard
246 curve for para-nitrophenol (pNP) was included in each assay and used to establish the amount of
247 product pNP produced (mM) and thus, enzyme activity. TRAP and ALP activities were
248 normalized using bone dry weight and expressed as nmol pNP.min^{-1}.mg^{-1}.

249 **Calcium and ash content in bone**

250 Individual crushed vertebrae samples (n=10/group), cleaned of muscle, were dried at
251 50ºC until each registered a constant weight (to the nearest 0.1 mg) in three independent
252 measurements (approximate drying time 48 hours). Ash content in vertebrae was determined by
253 incinerating dried samples at 550ºC for 14 hours and then cooling the ashes in a desiccator and
254 determining their weight (precision of 0.1 mg). The ash content was normalized by the dry mass
255 of bone and expressed as mg. Ashes were then digested for 24 hours with 70% nitric acid (200
256 µl.mg^{-1} ash) and their calcium content determined using an Agilent Microwave Plasma-Atomic
257 Emission Spectrometer (MP-AES), model 4200 (Agilent Technologies, USA). Calcium
258 concentrations were measured in each digested sample, diluted 1:1000 in acidic water (5% nitric

259 acid), by comparison with a standard curve ranging between 0.5 and 10 ppm (parts per million)
260 of calcium (Agilent Calibration Mix Majors 6610030700). Running parameters for MP-AES were
261 pump rate 15 rpm, sample uptake time 70 sec, rinse time 40 sec, stabilization time 15 sec, with 5
262 replicate readings and the selected options "fast pump during uptake" and "rinse time fast pump"
263 in mode "on". Calcium contents were measured at a wavelength of 393 nm and then expressed as
264 $\mu mol.mg^{-1}$.

265 **Analysis of gene expression by quantitative real-time PCR (qPCR)**

266 Total RNA was extracted from crushed vertebrae (n=10/group) using a Maxwell 16
267 System (Promega, USA) and following the manufacturer's instructions. The concentrations and
268 quality of the extracted RNA were determined using a NanoDrop1000 Spectrophotometer
269 (Thermo Fisher Scientific, USA) and by electrophoresis on 0.8% agarose gels. To eliminate
270 genomic DNA the total RNA (2–9 µg) was treated with DNase using a DNA-free kit (Ambion,
271 UK). cDNA synthesis was carried out in a 20 µL reaction volume containing 500 ng of DNase-
272 treated RNA, 200 ng of random hexamers (Jena Biosciences, Germany), 100 U of RevertAid
273 reverse transcriptase (Fermentas, Thermo Fisher Scientific, USA), 8 U of RiboLockRNase
274 Inhibitor (Fermentas) and 0.5 mM dNTPs (GE Healthcare, Spain). The reaction mixture was
275 incubated for 10 min at 20ºC followed by 50 min at 42ºC and the enzyme inactivated by heating
276 for 5 min at 72ºC.

277 Quantitative Real-Time PCR (qPCR) was used to analyze the mRNA expression of a suite
278 of genes characteristic of the bone matrix and associated with its activity, osteocalcin (*ocn*), and
279 mimecan/osteoglycin 1 and 2 (*ogn1* and *ogn2*) and other genes indicative of a change in bone
280 tissue regulation, *igf1*, *gr*, *trα* and *trβ* (Collins et al., 1998; Moutsatsou et al., 2012; Sbaihi et al.,
281 2007). Duplicate reactions for each individual cDNA were prepared in 15 µL, containing 10 ng
282 of cDNA, 300 nM of each specific primer and 1 times final concentration of EvaGreen (Sso Fast
283 Eva Green Supermix, Bio-Rad Laboratories, USA). In the case of the reference gene, *18s*, only
284 0.01 ng cDNA was used. PCR reactions were carried out in a StepOnePlus qPCR thermocycler
285 and data was analysed with StepOne software v2.2 (Applied Biosystems, UK). qPCR cycling
286 conditions were 30 sec at 95ºC, 40 cycles of 5 sec at 95ºC and 10 sec at 60ºC followed by a final
287 melt curve between 60 and 95ºC, which gave single products/dissociation curves in all reactions.
288 Specific primers for each transcript were designed using Primer Premier 5.0 software (Premier
289 Biosoft Int., CA, USA). Primer sequences, amplicon size, amplicon melting temperature, reaction
290 efficiency, R^2 and the accession number of genes are listed in Table 2. Standard curves relating
291 amplification cycle to initial template quantity (in copy number, calculated as in Vieira et al.,
292 2012) were generated using serial dilutions of purified and quantified target amplicons. All
293 amplicons were sequenced to confirm qPCR specificity. Control reactions included a no-template
294 control and a cDNA synthesis control (reverse transcriptase omitted).

295 Several reference genes were tested (beta actin, ribosomal protein S18 and 18S ribosomal
296 RNA subunit) and *18s* was selected as it did not vary significantly between cDNA samples of
297 vertebrae from adults used in the cold challenge experiment. Relative expression levels were
298 calculated by dividing the detected copy number of the target genes by the reference gene. Results
299 are expressed as Log2 Fold Change and were calculated relative to the control group, which was
300 defined as the experimental animals obtained from larvae maintained at 18ºC from egg until the
301 larvae-juvenile transition, since this is the temperature regime frequently used for gilthead sea
302 bream larval rearing (Mozes et al., 2011). The comparisons made and the strategy for statistical
303 analysis is indicated below.

304 **Statistical analysis**

305 All statistical analysis was performed using the SPSS 22.0 software package (SPSS Inc.,
306 Chicago, IL, USA) with statistical significance taken at $P<0.05$. No significant tank effects were
307 detected and the results for the samples from the duplicate tanks were pooled for statistical
308 analysis (student's t-test). Two-way analysis of variance (two-way ANOVA) was used to assess
309 the interaction between thermal history and the water temperature during the cold challenge
310 experiment for each of the parameters analyzed (biometric, plasma, vertebrae TRAP and ALP,
311 vertebrae minerals and gene expression). Bonferroni adjustment was used for pairwise
312 comparisons to identify any significant differences between different thermal history groups
313 maintained at 23±1ºC or between different thermal history groups exposed to a cold challenge
314 (13±1ºC) for each of the parameters analyzed. Any significant differences between fish from the
315 same thermal regime maintained at 23±1ºC or exposed to a cold challenge (13±1ºC) was also
316 identified. Dunnett's pairwise comparison was conducted for qPCR results to identify any
317 significant difference between the control group (LT at 23±1ºC) and the other groups. Log10
318 transformation of the data was used whenever necessary to achieve either normal distribution or
319 equal variance assumptions. Data is presented as mean ± standard error of the mean (s.e.m.),
320 unless otherwise stated.

321 **Results**

322 **Biometric parameters**

323 Two-way ANOVA revealed that body weight and length of adult fish were affected by
324 thermal history ($P<0.001$), whereas condition factor K was affected by cold temperature challenge
325 ($P<0.01$, Table 1). HSI was affected by both thermal history and the temperature challenge
326 ($P<0.001$) and also by the interaction between both factors ($P<0.01$).
327 Fish from LHT group were significantly heavier and larger ($P<0.001$) than fish from other
328 thermal groups irrespective of water temperature. However, no significant covariation was

329 detected between body mass and the other physiological parameters monitored. Fish exposed to
330 a cold temperature challenge (13±1ºC) for 15 days had a significantly higher HSI (*P*<0.01, LT
331 and LHT; *P*<0.001, HLT and HT) than those maintained at 23±1ºC irrespective of their thermal
332 history. The condition factor K did not differ significantly between fish with the same thermal
333 history maintained at 23±1ºC or exposed to 13±1ºC. The exception was the LT fish in which K
334 was significantly (*P*<0.05) lower in the cold challenged (13±1ºC) group relative to the fish
335 maintained at 23±1ºC. No significant differences were found by the end of the experiment in the
336 weight or length of fish from the same thermal history maintained at 23ºC or exposed to 13ºC for
337 15 days.

338 **Characterization of the physiological response to cold stress**

339 *Plasma cortisol*

340 Two-way ANOVA revealed that plasma cortisol levels were significantly affected by
341 thermal history (*P*<0.001), by temperature challenge (*P*<0.001) and by the interaction between
342 these two factors (*P*<0.001, Fig. 2). However, no significant differences in plasma cortisol
343 concentrations were detected between LT, LHT, HT and HLT fish maintained at 23±1ºC,
344 although values ranged between 60.3±16.6 ng.mL^{-1} (HT) and 85.0±11.8 ng.mL^{-1} (LHT; Fig. 2).
345 However, at 13±1ºC HLT fish had significantly (*P*<0.001) higher levels of plasma cortisol
346 (108.4±25.24 ng.mL^{-1}) relative to LT, LHT and HT fish, and LT fish had significantly (*P*<0.05)
347 higher levels of plasma cortisol (35.0±12.2 ng.mL^{-1}) relative to the LHT fish (1.5±0.36 ng.mL^{-1}).
348 The LT, LHT and HT fish exposed to a cold challenge (13±1ºC) had significantly (*P*<0.01) lower
349 levels of plasma cortisol than the equivalent group of fish maintained at 23±1ºC.

350 *Plasma glucose*

351 Two-way ANOVA revealed that the interaction between thermal history and temperature
352 challenge significantly (*P*=0.02) affected the concentration of plasma glucose (Fig. 2).
353 Comparison of plasma glucose levels of the LT, LHT, HT and HLT fish maintained at 23±1ºC
354 revealed no significant differences between groups. Similarly, the plasma glucose concentrations
355 in LT, LHT, HT and HLT fish exposed to a cold challenge (13±1ºC), did not differ. Comparison
356 of fish with the same thermal history revealed that the concentration of plasma glucose increased
357 significantly (*P*<0.01) in the HLT group exposed to a cold temperature challenge (7.8±0.43
358 mmol.L^{-1}) relative to those maintained at 23±1ºC (5.9±0.49 mmol.L^{-1}).

359 *Plasma Na^+, K^+, protein and osmolality*

360 Na^+ and K^+ plasma concentrations were significantly (*P*<0.05 and *P*<0.01, respectively)
361 affected by thermal history and the cold temperature challenge, whereas protein and osmolality

362 were only significantly (P<0.001 and P<0.05, respectively) affected by a cold challenge (Table 363 3). Plasma Na^+ was also significantly (P<0.001) affected by the interaction between both factors. 364 Plasma Na^+, K^+, protein and osmolality in LT, LHT, HT and HLT fish maintained at 23±1ºC were 365 not significantly different (Table 3). Comparison of LT, LHT, HT and HLT fish exposed to a cold 366 challenge (13ºC) revealed that the HLT fish had significantly (P<0.01) lower plasma Na^+ and K^+. 367 No significant differences were detected in the concentration of plasma Na^+, K^+, protein and 368 osmolality when they were compared to fish with the same thermal history maintained at 23ºC or 369 exposed to a cold challenge (13±1ºC). The exception was the HLT fish in which the concentration 370 of plasma Na^+ and K^+ was significantly (P<0.001 and P<0.01, respectively) lower in cold 371 challenged fish relative to those maintained at 23±1ºC. In the HLT group, plasma osmolality and 372 protein were significantly (P<0.05) higher in the cold challenged fish relative to those maintained 373 at 23±1ºC. Plasma calcium levels were not affected by thermal history or by a low temperature 374 challenge.

375

376 **Characterization of bone metabolism in response to cold stress**

377 *TRAP and ALP activity in vertebrae*

378 Two-way ANOVA indicated that a cold challenge significantly (P<0.001) impacted on 379 the TRAP and ALP activities in bone, and that the ALP activity was also significantly (P<0.01) 380 affected by thermal history (Fig. 3). Comparison of TRAP activity in the haemal vertebrae of the 381 LT, LHT, HT and HLT fish maintained at 23±1ºC revealed no significant differences between 382 groups. The ALP activity in the vertebrae of LT and HLT fish was significantly (P=0.009) higher 383 than the HT fish maintained at 23±1ºC. The cold challenge (13±1ºC) failed to cause a significant 384 difference in either ALP or TRAP activities when LT, LHT, HT and HLT fish were compared. 385 Comparison of fish with the same thermal history revealed that a cold challenge caused a 386 significant (P=0.001) decrease in the ALP activities of the LT and HLT groups relative to fish 387 maintained at 23±1ºC. Similarly, the TRAP activity of the vertebrae of fish with the same thermal 388 history that were exposed to a cold challenge was significantly (P<0.01) lower in the LT, HLT 389 and HT groups relative to those maintained at 23±1ºC. No significant differences in the 390 TRAP/ALP ratio (data not shown) were detected in fish with the same thermal history that were 391 maintained at 23±1ºC or exposed to a cold challenge of 13±1ºC for 15 days.

392 *Calcium content in vertebrae*

393 Two-way ANOVA revealed a significant (P=0.02) effect of thermal history on the 394 calcium content of haemal vertebrae (Table 4). Comparison of the calcium content in the vertebrae 395 of the LT, LHT, HT and HLT fish maintained at 23±1ºC revealed no significant differences

396 between groups. Comparison of LT, LHT, HT and HLT fish exposed to a cold challenge (13±1ºC)
397 revealed that the LHT fish had a significantly (*P*=0.03) higher calcium content than fish of the
398 LT regime. No significant differences in ash content of vertebrae were detected in fish with the
399 same thermal history that were maintained at 23±1ºC or exposed to 13±1ºC for 15 days (Table
400 4).

401 **Gene expression in bone in response to cold stress**

402 *Transcripts of the bone matrix in vertebrae*

403 Two-way ANOVA revealed that cold temperature challenge significantly (*P*<0.01)
404 modified the expression of bone matrix transcripts, *ocn* and *ogn1* (Fig. 4). No significant
405 differences in *ocn*, *ogn1* and *ogn2* were identified in the vertebrae of the LT, LHT, HT and HLT
406 fish maintained at 23±1ºC. Transcripts of *ogn1* and *ogn2* were significantly (*P*<0.05) up-regulated
407 in vertebrae of HLT relative to the LHT fish at 13±1ºC. In vertebrae of the cold challenged HLT
408 fish, *ocn* was significantly (*P*<0.05) up-regulated relative to the HT fish. Comparison of vertebrae
409 from fish with the same thermal history indicated that a cold challenge caused a significant
410 (*P*<0.05) down-regulation of *ogn1* and *ogn2* transcripts in the LHT fish, but no differences were
411 detected in any of the other groups. In the LHT and HT groups, a cold challenge caused a
412 significant (*P*<0.05) down-regulation of *ocn* transcripts in vertebrae compared to the same group
413 maintained at 23±1ºC. Comparison of gene expression in vertebrae from cold challenged LT,
414 HLT, LHT and HT fish with the LT group at 23±1ºC (the temperature frequently used for larval
415 culture, Mozes et al., 2011), revealed significant (*P*<0.05) down-regulation of transcripts for *ocn*
416 in the HT fish and *ogn1* in the LT and LHT fish.

417 *Transcripts of regulatory factors in vertebrae*

418 Two-way ANOVA revealed that when fish with different thermal histories were exposed
419 to a cold challenge, *gr* and *igf1* expression in vertebrae was modified due to a significant
420 (*P*=0.001) interaction between thermal history and low temperature challenge (Fig. 5). Similarly,
421 *trα* and *trβ* expression was affected by the significant interaction (*P*<0.01 and *P*=0.01,
422 respectively) that occurred between thermal history and the cold temperature challenge (Fig. 5).
423 *Gr* and *trα* expression was also significantly (*P*<0.001) affected by temperature challenge.
424 Comparison of the transcript abundance of *igf1, trα* and *trβ* in vertebrae of LT, LHT, HT and
425 HLT fish kept at 23±1ºC revealed they were similar irrespective of their thermal histories. In
426 contrast, *gr* was significantly (*P*<0.05) lower in vertebrae of the HLT fish relative to the LHT fish
427 maintained at 23±1ºC. In LT, LHT, HT and HLT fish exposed to a cold challenge (13±1ºC), *gr*
428 and *igf1* expression in vertebrae was significantly (*P*<0.01) lower in the LHT relative to HLT
429 fish.

430 Comparison of fish with the same thermal history exposed to a cold temperature challenge
431 revealed significant (P<0.05) down-regulation of *gr*, *igf1*, *trα* and *trβ* in vertebrae from the LHT
432 fish relative to the fish maintained at 23±1ºC. In the HT group, a cold challenge caused a
433 significant (P<0.05) down-regulation of *gr* and *trα* in vertebrae relative to the same group
434 maintained at 23±1ºC.

435 Comparison of gene expression in vertebrae of the LT group maintained at 23±1ºC
436 (control fish) and the LT, HLT, LHT and HT fish that were exposed to cold challenge revealed
437 significant (P<0.05) down-regulation of *igf1* expression in the LT and LHT fish, significant
438 down-regulation of *gr*, *trα* and *trβ* in the LHT fish (P<0.05) and significant down-regulation of
439 *trα* in LT fish (P<0.05).

440

441 Discussion

442 This study is the first to investigate the effect of early thermal history on the response of
443 adult sea bream to a cold challenge and more specifically the potential change in bone activity
444 and the bones likely response to the endocrine system in fish from different thermal regimes.
445 When adult sea bream with different thermal histories were exposed to a cold water challenge
446 they had a different physiological response and overt differences in the stress axis was observed
447 during the study. Significant differences in plasma parameters like glucose, sodium, potassium,
448 osmolality, protein and cortisol occurred between the experimental groups even before cold
449 temperature exposure, suggesting that the early thermal regimes modified their physiology. The
450 HLT thermal regime had the greatest impact on plasma parameters and was significantly different
451 in adults of this group relative to the other thermal groups when they were challenged by a drop
452 in water temperature. The early thermal history also significantly influenced the responsiveness
453 of bone to a cold challenge (13±1ºC) and fish from the LHT treatment was the most different
454 from the other groups. In the LHT fish, a cold challenge caused a reduction in the relative
455 abundance of the bone ECM transcripts, osteocalcin and osteoglycin and also transcripts linked
456 with bone responsiveness, suggesting thermal imprinting modified the bone.

457 Thermal challenge and somatic indexes

458 In line with previous reports, a decrease in water temperature was associated with a
459 significant reduction in feed intake, which is one of the first signs of cold stress (Tort et al., 2004).
460 However, in the present study irrespective of thermal history, the reduction in feed intake as a
461 consequence of a drop in water temperature did not affect body weight or K, which was similar
462 to the matched controls maintained at 23±1ºC. These results are in line with other studies of cold
463 challenged sea bream, in which body weight was not affected by low water temperatures (Tort et
464 al., 2004), although HSI was increased as a consequence of the failure to mobilize fat stores (Ibarz

465 et al., 2007; Ibarz et al., 2005). We propose that the maintenance of body weight and K in the
466 present study indicates that sea bream were able to adjust their metabolism to compensate for the
467 effects of a short-term (15 days) cold challenge as it has been shown for other fish (Hochachka
468 and Somero, 1984). Our results contrast with previous studies in which sea bream were unable to
469 maintain their body mass presumably because the water temperature in previous studies was
470 dropped to below 10ºC and the fish totally stopped feeding (Ibarz et al., 2003). Overall, our results
471 suggest that thermal imprinting did not influence the capacity of the sea bream to compensate
472 their metabolism when water temperature was reduced.

473 **Thermal challenge as a stressor**

474 In the present study, the cortisol response at different time points during the experiment
475 was not established and so it was not possible to confirm if a drop in water temperature caused a
476 transient peak in cortisol as previously reported in the gilthead sea bream (Rotllant et al., 2000)
477 and the Atlantic cod (Gadus morhua, Staurnes et al., 1994). Furthermore, increased plasma
478 glucose (a secondary stress marker, Pottinger and Pickering, 1997) was only observed in the HLT
479 group when fish were exposed to 13ºC for 15 days. However, the results from several previous
480 studies suggest that the development of hyperglycemia in response to a cold challenge is variable
481 in this species (Sala-Rabanal et al., 2003; Tort et al., 2004; Vargas-Chacoff et al., 2009). Notably,
482 the only group that was hyperglycemic in our experiments (the HLT group) was also the group
483 that had significantly higher plasma cortisol (108.4±71.4 ng.mL^{-1}). A positive correlation between
484 plasma cortisol and glucose has been previously reported in Atlantic cod under cold stress
485 (Staurnes et al., 1994). By the end of the cold challenge, a drastic reduction in plasma cortisol
486 occurred in the LT, LHT and HT groups relative to the same thermal group maintained at 23ºC,
487 which is in agreement with the results of previous studies in the gilthead and silver sea bream
488 (Deane and Woo, 2005; Rotllant et al., 2000). The results of the present study indicate that in the
489 gilthead sea bream thermal imprinting modified the cortisol response in adults when they were
490 exposed to a cold challenge, presumably through modifications in the stress axis. In fact, in a
491 previous study, exposure to an acute stress challenge of slightly older fish (9 months old) from
492 the same population of fish revealed that thermal imprinting caused significant changes in the
493 central stress axis (Mateus et al., in press).
494 The reference resting values for plasma cortisol in gilthead sea bream are between 1-10
495 ng.mL^{-1} and for chronic (around 33±34.1 ng.mL^{-1}) and acute stress (162±101.8 ng.mL^{-1}) (Tort, *et*
496 *al*, 2011) are significantly higher. Surprisingly, plasma cortisol levels in fish maintained at 23ºC
497 under standard experimental conditions were those characteristic of a stress response. The
498 elevated cortisol levels may have been a result of the acute stress of capture and handling (Laidley
499 and Leatherland, 1988; Molinero et al., 1997) even though we endeavored to minimize stress
500 during sampling. The results tend to suggest that the stress response in the 13ºC challenged LT,

501 LHT and HT groups was suppressed, although the mechanism by which this occurred was not
502 established in the present study and will be a target for future studies.

503 The present study confirmed the hypothesis raised by others (Beitinger et al., 2000;
504 Somero, 2005) that thermal history influences thermal tolerance in adult fish. To our knowledge,
505 only one other study has investigated the effects of thermal history on the thermal tolerance of
506 adult fish and it involved exposing zebrafish to high water temperatures (Schaefer and Ryan,
507 2006), but did not assess how the challenge modified physiological and endocrine systems. The
508 results of our study confirm the general notion that non-lethal stress in early life may modify
509 whole animal physiology and favor improved acclimation to stressors in later life (Jones, 2012).
510 However, the results of our study indicate that the characteristics and timing of the stress, in this
511 case temperature, may play a crucial role in determining the impact on adult physiology. For
512 example, the physiological response of the LHT and HLT groups of gilthead sea bream to a low
513 temperature challenge differed. At the end of the cold challenge, the HLT fish had higher glucose
514 and cortisol levels, while the LHT fish had a suppressed cortisol response that reached the resting
515 levels and plasma glucose levels were unchanged, which may suggest that LHT fish were more
516 apt at acclimating to a low water temperature. This supports the notion that embryonic stage may
517 be a critical window of increased susceptibility to temperature induced changes in fish
518 development (Scott and Johnston, 2012; Skjærven et al., 2011).

519 **Thermal challenge and plasma parameters**

520 A notable feature in the thermally imprinted fish was that in two independent experiments
521 with 7 month old (present study) and 9 month old (Mateus et al. in press) thermally imprinted sea
522 bream the results for the plasma chemistry under control conditions (23±1ºC) were similar. This
523 suggests that thermal imprinting caused a persistent physiological change that was not affected
524 by age or time of year.

525 A drop in water temperature has previously been reported to produce an imbalance in
526 plasma chemistry, which can impact on a number of processes including metabolism and
527 osmoregulation (Donaldson et al., 2008; Ibarz et al., 2010b; Rotllant et al., 2000). Previous studies
528 have revealed that cold water challenge in gilthead sea bream caused an imbalance in plasma ions
529 and most notably a reduction in plasma calcium, sodium and potassium levels (Gallardo et al.,
530 2003; Rotllant et al., 2000; Sala-Rabanal et al., 2003; Vargas-Chacoff et al., 2009) and a
531 significant increase in osmolality in juvenile turbot (*Scopthalmus maximus*, Imsland et al., 2003)
532 and in tilapia hybrids (*Oreochromis mossambicus* x *O. urolepis hornorum*, Sardella et al., 2004).
533 The modified plasma ion profile in cold challenged sea bream has been linked to a change in their
534 osmoregulatory capacity, resulting from a change in the morphology of the gill epithelium and a
535 drastic reduction in gill, intestine and kidney Na^+/K^+-ATPase activity (Ibarz et al., 2010b).
536 Overall, the results for plasma chemistry in the present study suggest that thermal imprinting had

537 differing consequences for the osmoregulatory response to cold challenge in the gilthead sea
538 bream. In particular, the thermal regimes associated with least change in plasma chemistry in
539 response to a cold challenge was the LT and LHT group, while in contrast, the HLT group suffered
540 a significant reduction in plasma sodium and potassium levels and a significant increase in
541 osmolality relative to the matched group maintained at 23ºC. The mechanism by which thermal
542 imprinting modified plasma chemistry was not established in the present study, but may result
543 from the changes induced by temperature in the developmental events occurring during embryo
544 and early larval development (Yúfera et al., 2011).

545 Plasma levels of total protein were also modified in fish exposed to a cold challenge and
546 the HLT and HT groups at 13ºC had significantly higher plasma protein levels than those of fish
547 from the same thermal history maintained at 23ºC. Field based (Guijarro et al., 2003; Vargas-
548 Chacoff et al., 2009) and laboratory studies (Gallardo et al., 2003) have previously reported
549 increased total plasma protein concentrations during winter or under lower temperatures,
550 respectively, as a result of increased β_2- and γ-globulins (Cataldi et al., 1998; Gallardo et al.,
551 2003). In the present study, only total plasma protein was measured and it remains to be
552 established if the increase in protein was linked to an increase in the γ-globulins fraction as
553 previously reported (Gallardo et al., 2003). Nonetheless, the significant increase in plasma protein
554 in the HT and HLT group exposed to a cold challenge raises the possibility that early thermal
555 history may modify the immune response in adult fish (Bizuayehu et al., 2015).

556 **Thermal challenge and bone homeostasis**

557 To evaluate the impact of early thermal history on bone remodeling in adult sea bream
558 maintained under optimal culture temperatures (23ºC), we focused on the mineral content, the
559 activity of the enzymes ALP and TRAP (Dimai et al., 1998; Persson et al., 1995), indicators of
560 osteoblast and osteoclast activity, respectively, and typical transcripts of the bone. Transcripts
561 included those encoding ECM proteins, such as osteocalcin (OCN), a protein extremely abundant
562 in the bone ECM that is a marker of late stage osteoblast differentiation, that is essential for
563 mineralization/remodeling (Fraser and Price, 1988; Karsenty and Oury, 2012; Lee et al., 2007)
564 and osteoglycin (OGN1/2), a small leucine-rich proteoglycan found in the extracellular matrix of
565 connective tissue, which is an osteoinductive factor in cows (Bentz et al., 1989; Iozzo, 1997) and
566 is associated with osteoblast differentiation (Kukita et al., 1990; Tanaka et al., 2012). Thermal
567 history did not substantially affect basal bone homeostasis in unchallenged gilthead sea bream as
568 the abundance of ECM transcripts and hormones receptors were similar in all experimental
569 groups. The exception was the ALP enzymatic activity, which was much lower in the HT fish,
570 suggesting their bone remodeling may be modified relative to the other fish, although the
571 reduction of ALP in HT was not linked to modified plasma cortisol, a factor known to suppress
572 ALP in humans (van Straalen et al., 1991).

573 The vertebral bone in gilthead sea bream from different thermal histories had a different
574 response to a cold challenge and the enzymatic activities of TRAP and ALP, and ECM and
575 hormone receptor transcript abundance were modified. The reduction in temperature associated
576 with cold challenge caused a simultaneous reduction in ALP and TRAP enzyme activity in fish
577 of LT and HLT groups. However, only fish from the LT group also had a decrease in bone calcium
578 content and a significant down-regulation of *ogn1*, which in other studies has been shown to be
579 indicative of modified bone remodeling in fish (Pombinho et al., 2004) and rat (Goto and
580 Tsukamoto, 2003). Although in the LHT group exposed to 13ºC ALP and TRAP were not
581 significantly modified relative to the matched group at 23ºC, *ocn* and *ogn1/2* were significantly
582 down-regulated, which is in line with the results of previous studies on fasted sea bream (Vieira
583 et al., 2013) and type I diabetic mice (Botolin et al., 2005). These results may suggest that later
584 stages of osteoblast differentiation were suppressed, while earlier stages were unaffected. If the
585 changes observed in bone from fish with different thermal histories, arose from epigenetic
586 mechanisms was not established in this study. However, evidences exist that temperature during
587 early development causes epigenetic modulation in the genome in teleosts (Bizuayehu et al., 2015;
588 Campos et al., 2014). Furthermore, in Atlantic cod reared at different temperatures after hatching
589 the expression of miRNAs associated with bone activity was modified (Bizuayehu et al., 2015),
590 and suggests a possible mechanism by which early rearing temperature can influence adult bone.

591 Bone is an emerging endocrine tissue (Blair et al., 2008) and also a target for a number
592 of endocrine hormones, such as glucocorticoids, thyroid hormone and insulin like growth factor
593 that regulate its turnover (Robson et al., 2002). The effect of a cold challenge on the
594 responsiveness of bony tissue in ectotherms and particularly those with different early thermal
595 histories has never been studied. Candidate transcript abundance was similar in all experimental
596 groups at 23ºC, suggesting thermal imprinting did not appear to modify basal bone metabolism
597 in adult sea bream. However, thermal imprinting changed the response of bone to a drop in water
598 temperature and *igf1*, associated with growth and bone turnover (Collins et al., 1998; Gabillard et
599 al., 2005; Ono et al., 1996), *trα* and *trβ*, associated with bone resorption (Blair et al., 2008; Sbaihi
600 et al., 2007) and *gr* that mediates the effects of cortisol (Moutsatsou et al., 2012), were all
601 significantly down-regulated in the LHT group vertebral bone. These results suggest that a drop
602 in water temperature impairs the responsiveness of bone by repressing the transcription of these
603 genes (Abbas et al., 2012; Larsen et al., 2001) and that this in turn impairs bone remodeling
604 (Suzuki and Hattori, 2002). It would be of interest to directly measure the change in bone ECM
605 proteins to assess the impact of thermal history and cold challenge on vertebral bone mass, but
606 since neither antisera or assays are currently available for fish, this was not possible. Nonetheless,
607 an intriguing observation was that the groups with the most significant down-regulation of bone
608 matrix transcripts (LHT, LT and HT) also had the most notable down-regulation of *gr*, *igf1*, *trα*

609 and *trβ*. Although a simultaneous decrease in TRAP and ALP activity was detected in HLT group,
610 no modification was identified in bone calcium content and ECM transcripts relative abundance
611 which may be justified by an unchanged endocrine response in the bone of fish of the HLT group.
612 This observation is in line with previous studies which have revealed that disruption of endocrine
613 signaling including thyroid (Sbaihi et al., 2007; Takagi et al., 1994) and cortisol in fish (Sbaihi et
614 al., 2009) and mice (Sher et al., 2006) modifies bone cell responsiveness to regulatory factors.
615 Overall, although thermal imprinting failed to modify bone metabolism and responsiveness in
616 optimal ambient water temperatures, it did modify the response of bone to a cold challenge. Future
617 studies should be directed at establishing the epigenetic mechanisms underlying this response.

618 List of symbols and abbreviations

619 GR, glucocorticoid receptor;
620 HLT, high-low temperature;
621 HSI, hepatosomatic index;
622 HT, high temperature;
623 IGF1, insulin-like growth factor 1;
624 K, condition factor;
625 LHT, low-high temperature;
626 LT, low temperature;
627 OCN, osteocalcin;
628 OGN, mimecan/osteoglycin.
629 pNP, para-nitrophenol;
630 pNPP, para-nitrophenyl phosphate;
631 TRα/β, thyroid receptors α or β;

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637 Competing interests

638 The authors declare no competing or financial interests.

639 Authors' contributions

DMP conceived and planned the project. EG, AKB, and EA ran the trials with the fish and the experimental sampling. RC was involved in sorting out samples, registering, changing solutions and maintained all the material under appropriate conditions. APM performed the practical work including plasma analyses and molecular biology. DMP and APM analyzed and interpreted the data and drafted the manuscript. PP coordinated bone mineral content analyses. PP, RC, EG, AKB and EA revised it critically for important intellectual content. All authors have given their final approval of the version to be published.

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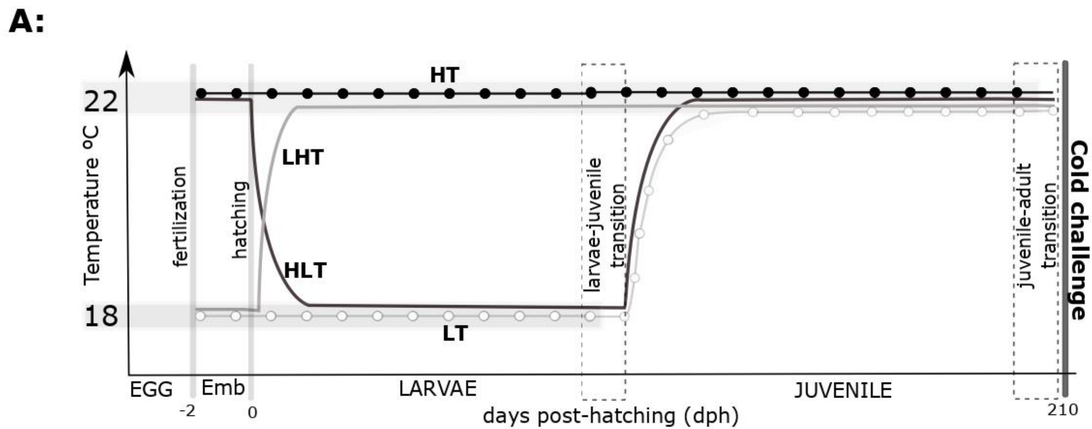
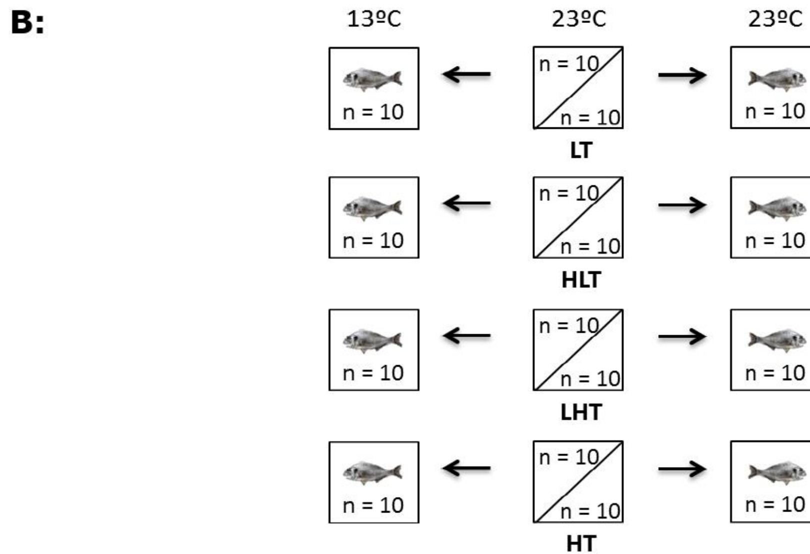
992 **Figure and Figure Legends**

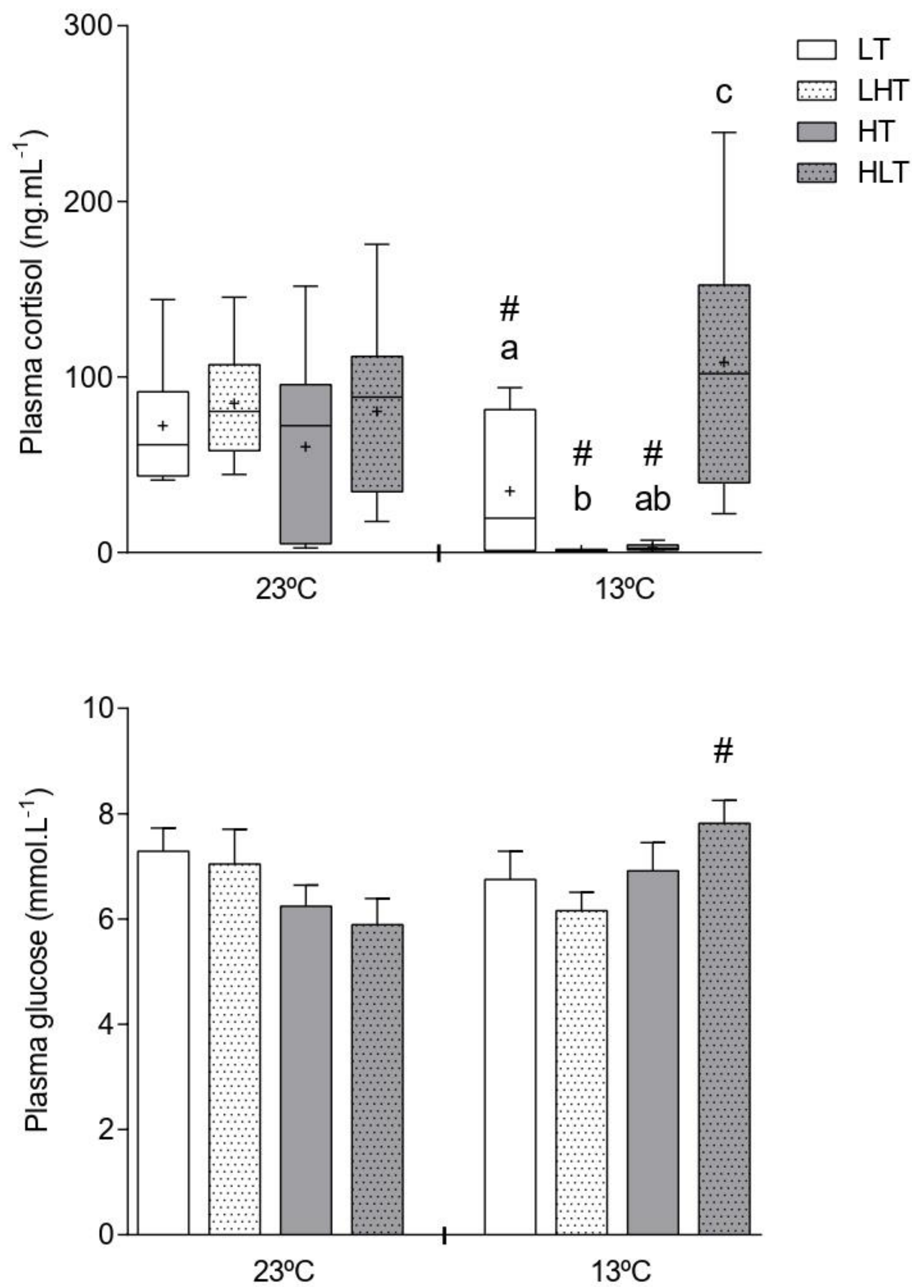




993

994 **Fig. 1.** Schematic representation of the temperature regimes that gilthead sea bream were exposed
995 from egg fertilization to larvae-juvenile transition until the cold challenge. A: Four temperature
996 treatments (thermal groups) were generated, two with constant temperatures (LT, low temperature
997 [18-18ºC] and high temperature, HT [22-22ºC]) and two with variable temperatures during the
998 egg incubation phase and larval rearing (HLT [22-18ºC] and LHT [18-22ºC]). Fish from all
999 thermal groups were maintained at a common temperature (22±1ºC) from the larvae-juvenile
1000 transition (when the body was covered with scales) for 7 months until the beginning of the cold
1001 challenge. B: The cold challenge was performed by randomly dividing each group of fish for a
1002 thermal regime into two groups. The control group was maintained in replicate tanks at 23ºC and
1003 the cold group was maintained in replicate tanks at 13ºC (n=10/thermal history group). Fish were
1004 subjected to these temperatures for 15 days until sampling.

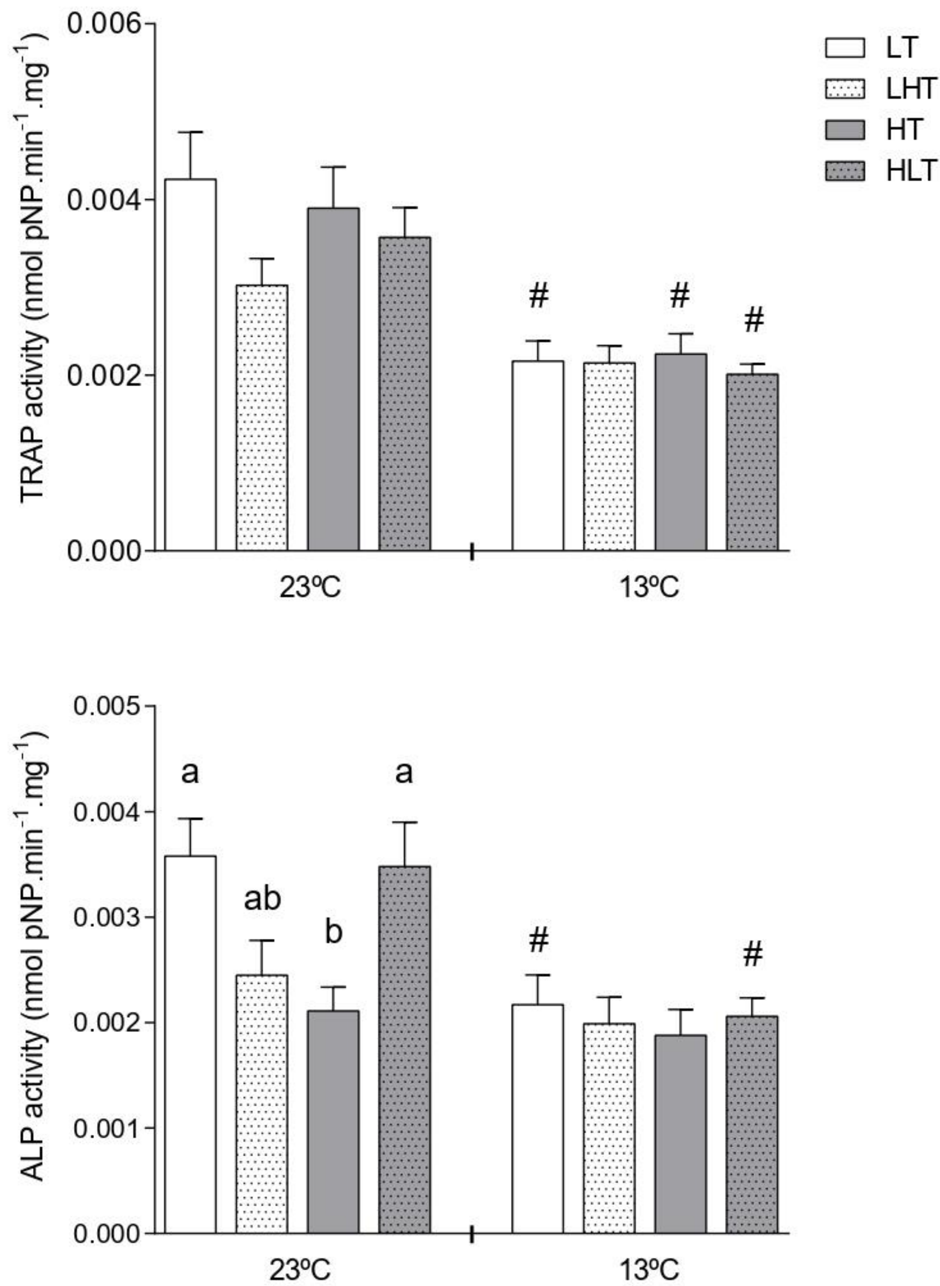
1005



1006

1007 **Fig. 2.** Cortisol and glucose plasma levels. The stress related parameters were analyzed in plasma
1008 samples from sea bream maintained in replicate tanks under control conditions (23ºC,
1009 n=10/thermal history) or under a cold challenge (13ºC, n=10/thermal history group) for 15 days.
1010 The cortisol levels are plotted in a Tukey box plot and whiskers graph (with '+' representing the
1011 mean) and the results of glucose are shown as mean±s.e.m. of the groups with different thermal
1012 history: LT (18-18ºC); LHT (18-22ºC); HT (22-22ºC); HLT (22-18ºC). Different letters indicate
1013 significant differences exist for cortisol levels between fish with a different thermal history
1014 maintained at the same temperature. Cardinal (#) indicates significant differences exist on glucose

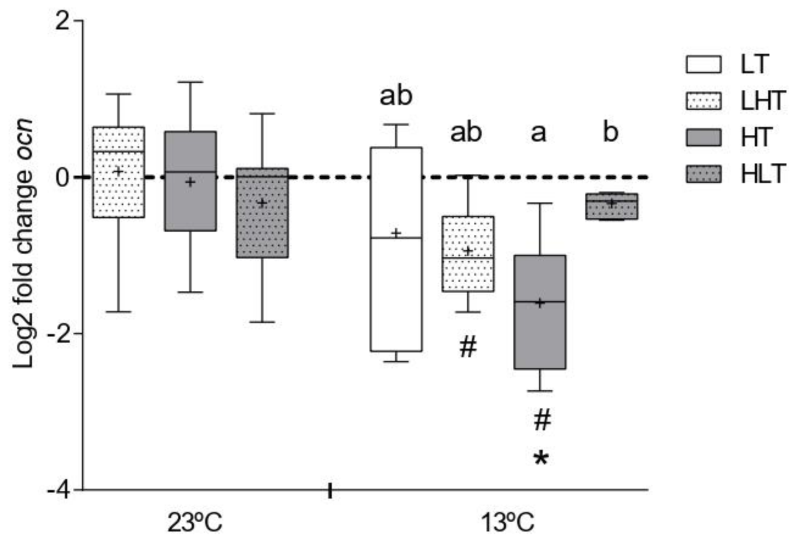
1015 levels between fish with the same thermal history maintained at different temperatures, 23ºC or
1016 13ºC. Two-way ANOVA; *P*<0.05.

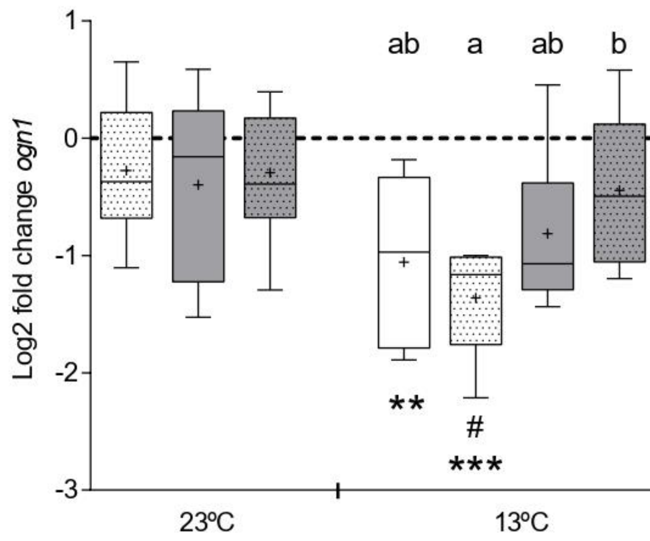


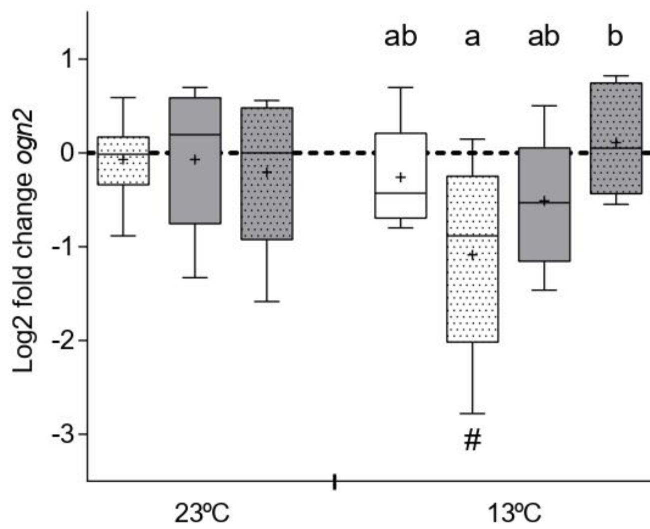
1017

1018 **Fig. 3.** Effects of low temperature challenge on biochemical markers of bone remodeling ALP
1019 and TRAP, measured in vertebral bone of sea bream with different thermal histories in the control
1020 (23ºC, n=10/thermal history) and cold challenged group (13ºC, n=10/thermal history group) 15
1021 days after acclimation to the temperatures. The results of the replicate tanks/ treatment were
1022 pooled for statistical analysis as no significant differences were found. The results obtained for
1023 fish from each thermal regime, LT (18-18 ºC); LHT (18-22 ºC); HT (22-22 ºC); HLT (22-18 ºC)

1024 are represented. Different letters indicate significant differences exist for ALP activity between
1025 fish with a different thermal history maintained at the same temperature. Cardinal (#) indicates
1026 significant differences existed between fish with the same thermal history maintained under
1027 control conditions 23ºC or exposed to a cold challenged, 13ºC. The results are shown as
1028 mean±s.e.m. of para-nitrophenol (pNP) production (nmol pNP.min.mg^{-1}). Statistical significances
1029 (by Two-way ANOVA) were set at $P<0.05$.

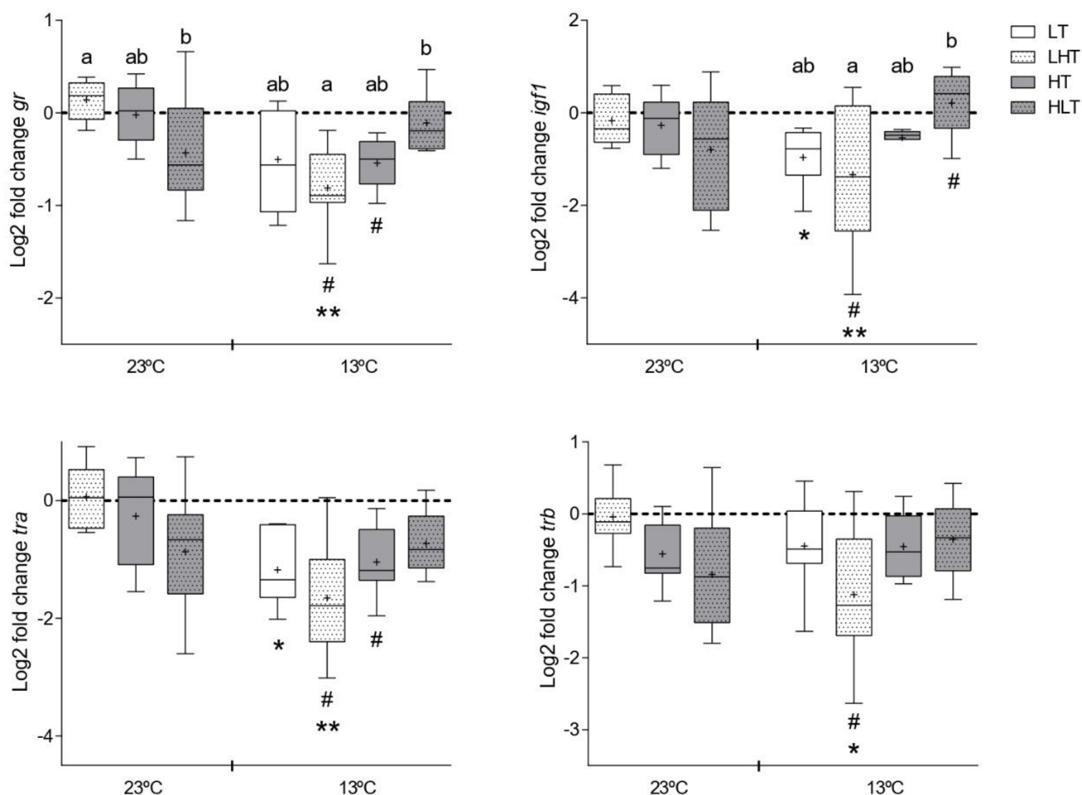






1030

1031 **Fig. 4.** Relative expression of transcripts associated with the bone matrix: *ocn*, *ogn1* and *ogn2*.
1032 Vertebral bone cDNA for each individual was analyzed by qPCR and normalized by the mean of
1033 *18s* expression: control group (23ºC, n=10/thermal history) and cold group (13ºC, n=10/thermal
1034 history group). Results for each thermal history group are expressed as Log2 Fold change relative
1035 to the LT group (thermal history 18-18ºC) maintained at 23ºC, defined as control and not
1036 represented (corresponds to the base line with fold change=0); results are represented in a Tukey
1037 box plot: LT (18-18ºC); LHT (18-22ºC); HT (22-22ºC); HLT (22-18ºC). '+' represents the mean.
1038 The results of the replicate tanks/ treatment were pooled for statistical analysis as no significant
1039 differences were found between them. Different letters indicate significant differences between
1040 the thermal groups maintained at the same temperature. Cardinal (#) indicates significant
1041 differences between fish with the same thermal history maintained under control conditions 23ºC
1042 or exposed to a cold challenged, 13ºC. Significant up-regulation or down-regulation relative to
1043 the control temperature (LT maintained at 23ºC) is denoted by: * $P<0.05$, ** $P<0.01$, *** $P<0.001$
1044 using Two-Way ANOVA.



1045

1046 **Fig. 5.** Relative expression of transcripts associated with endocrine responsiveness at low
1047 temperatures: *gr*, *igf1*, *trα* and *trβ*. Vertebral bone cDNA for each individual was analyzed by
1048 qPCR and normalized by the mean of *18s* expression: control group (23ºC, n=10/thermal history)
1049 and cold group (13ºC, n=10/thermal history group). Results for each thermal history group are

1050 expressed as Log2 Fold change relative to the LT group (thermal history 18-18ºC) maintained at
1051 23ºC, defined as control and not represented (corresponds to the base line with fold change=0);
1052 results are represented in a Tukey box plot: LT (18-18ºC); LHT (18-22ºC); HT (22-22ºC); HLT
1053 (22-18ºC). '+' represents the mean. The results of the replicate tanks/treatment were pooled for
1054 statistical analysis as no significant differences were found between them. Different letters
1055 indicate significant differences between the thermal groups maintained at the same temperature.
1056 Cardinal (#) indicates significant differences occurring between fish with the same thermal history
1057 maintained under control conditions 23ºC or exposed to a cold challenge, 13ºC. Significant up-
1058 regulation or down-regulation relative to the control (taken at the LT group maintained at 23ºC)
1059 was denoted by: * P<0.05, ** P<0.01 using Two-Way ANOVA.

1060 **Table 1:** Summary of body and liver weight combined (g), standard length (cm), HSI (%; 100 x
1061 [liver mass/body mass]) and condition factor (K; 100 x (body weight/total length^3), of gilthead
1062 sea bream exposed to different thermal regimes during egg and larval stages and then maintained
1063 at the control temperature, 23±1ºC (n=10/group, control) or exposed to a temperature drop to
1064 13±1ºC (n=10/group).

Thermal history	Weight (g)		Standard Length (cm)		HSI		K	
Water temperature	23ºC	13ºC	23ºC	13ºC	23ºC	13ºC	23ºC	13ºC
LT (18-18ºC)	110.7 ± 13.9^a	100.3 ± 6.8^a	14.7 ± 0.76^a	14.2 ± 0.55^a	1.6 ± 0.4	2.2 ± 0.4$^{a\ ***}$	3.88 ± 0.33	3.54 ± 0.24*
LHT (18-22ºC)	172.5 ± 21.4^b	179.1 ± 18.8^b	17.0 ± 0.93^b	17.1 ± 0.67^b	1.4 ± 0.2	1.8 ± 0.3$^{b\ **}$	3.62 ± 0.32	3.46 ± 0.24
HT (22-22ºC)	149.0 ± 16.9^c	143.2 ± 21.8^c	15.9 ± 0.79^c	15.7 ± 1.02^c	1.5 ± 0.2	2.6 ± 0.3$^{c\ ***}$	3.83 ± 0.07	3.67 ± 0.28
HLT (22-18ºC)	109.1 ± 21.7^a	123.3 ± 15.8^c	14.6 ± 0.98^a	15.1 ± 0.78^{ac}	1.3 ± 0.3	2.2 ± 0.3$^{a\ ***}$	3.82 ± 0.22	3.67 ± 0.54

1065 Different letters indicate significant differences exist for a given parameter between fish with a
1066 different thermal history maintained at the same temperature. Asterisks denote significant
1067 differences between fish with the same thermal history maintained at different temperatures, 23ºC
1068 or 13ºC: * $P<0.05$; ** $P<0.01$; *** $P<0.001$. The results are shown as mean±s.d.; Two-way
1069 ANOVA; $P<0.05$.

1070 **Table 2:** Primers used for gene expression analysis by quantitative RT-PCR. Gene name,
1071 accession number, primer sequence, amplicon length (bp), annealing temperature (T, ºC) and
1072 qPCR efficiency (%) and R^2 are indicated for each primer pair (F=forward and R=reverse primer).

Gene Name	Accession No.	Primer Sequence (5´ to 3´)	Amplicon (bp)	T (ºC)	Efficiency (%)	R^2
ocn	AF289506	F: TCCGCAGTGGTGAGACAGAAG R: CGGTCCGTAGTAGGCCGTGTAG	150	60	99	0.991
gr	DQ486890	F: CCATCACCTCTGCCGCATCTG R: CTGGAGGAACTGCTGCTGAACC	195	64	84	0.994
ogn1	KM603667	F: GAAGTCTCTCTTATTCACCTGT R: CAAAGGGTCACTGAAGTATCCA	138	60	100	0.997
ogn2	KM603668	F: TGTTATTCTCCCATGGATCCTG R: GATCCCCCGCTGCATCTGTGG	125	60	98	0.998
igf1	AY996779	F: TGTCTAGCGCTCTTTCCTTTCA R: AGAGGGTGTGGCTACAGGAGATAC	84	60	100	0.995
trα	AF047467	F: GAGGCCGGAGCCAAACAC R: GCCGATATCATCCGACAGG	124	60	102	0.988
trβ	AY246695	F: ACCGACTGGAGCCCACACAG R: CCTTCACCCACGCTGCACT	129	60	101	0.992
rps18	AM490061	F: AGGGTGTTGGCAGACGTTAC R: CTTCTGCCTGTTGAGGAACC	164	60	96	0.994
β-actin	X89920	F: CCCTGCCCCACGCCATCC R: TCTCGGCTGTGGTGGTGAAGG	94	60	86	0.994
18s	(Pinto et al., 2010)	F: TGACGGAAGGGCACCACCAG R: AATCGCTCCACCAACTAAGAACGG	82	60	93.6	0.992

1073

1074 **Table 3:** Changes in plasma total protein, sodium, potassium, osmolality and total calcium in
1075 gilthead sea bream with different thermal histories maintained at 23ºC or 13ºC for 15 days.
1076 Replicate tanks were used per treatment, but for statistical analysis the data / replicate were pooled
1077 as no significant differences were detected (23ºC, n=10/thermal history; 13ºC, n=10/thermal
1078 history).

Thermal History	Sodium (mM)		Potassium (mM)		Protein (mg.mL^{-1})		Osm (mmol.Kg^{-1})		Calcium (mmol.L^{-1})	
Water temperature	23ºC	13ºC	23ºC	13ºC	23ºC	13ºC	23ºC	13ºC	23ºC	13ºC
LT (18-18ºC)	199.9 ± 3.76	187.1 ± 2.20^{a*}	3.0 ± 0.21	2.6 ± 0.16^{ab}	24.1 ± 0.75	26.4 ± 0.98	364.4 ± 3.16	368.6 ± 6.79	4.2 ± 0.04	3.9 ± 0.28
LHT (18-22ºC)	184.4 ± 6.04	183.7 ± 4.02^{a}	2.7 ± 0.16	2.1 ± 0.18^{ab*}	25.3 ± 0.64	27.5 ± 1.03	364.5 ± 4.84	361.1 ± 3.67	4.0 ± 0.10	4.0 ± 0.12
HT (22-22ºC)	184.5 ± 2.99	184.2 ± 2.39^{a}	2.7 ± 0.21	2.7 ± 0.11^{a}	23.9 ± 0.77	28.6 ± 1.03***	361.1 ± 5.59	373.0 ± 6.18	4.0 ± 0.15	3.8 ± 0.15
HLT (22-18ºC)	198.6 ± 5.44	159.5 ± 3.91^{b***}	2.8 ± 0.21	2.0 ± 0.12^{b**}	24.7 ± 0.62	27.4 ± 0.92*	359.0 ± 3.35	374.6 ± 4.36*	4.0 ± 0.11	3.5 ± 0.24

1079 Different letters indicate significant differences exist for a given parameter between fish with a
1080 different thermal history maintained at the same temperature. Asterisks denote significant
1081 differences between fish with the same thermal history maintained at different temperatures, 23ºC
1082 or 13ºC: * P<0.05; ** P<0.01; *** P<0.001. The results are shown as mean±s.e.m.; Two-way
1083 ANOVA; P<0.05.

1084 **Table 4:** Calcium (µmol.mg^{-1}) and ash (mg) content of vertebral bone of gilthead sea bream with
1085 a different thermal history exposed to control conditions (23ºC, n=10/thermal history) or exposed
1086 to a temperature drop (13ºC, n=10/thermal history group) for 15 days after acclimation to the
1087 conditions.

Thermal History	Calcium (µmol.mg^{-1})		Ash (mg)	
Water temperature	23ºC	13ºC	23ºC	13ºC
LT (18-18ºC)	24.0 ± 0.86	20.6 ± 0.36[a]	0.26 ± 0.015	0.29 ± 0.010
LHT (18-22ºC)	25.2 ± 1.63	26.3 ± 1.59[b]	0.28 ± 0.015	0.31 ± 0.011
HT (22-22ºC)	22.4 ± 0.67	25.4 ± 1.48[ab]	0.30 ± 0.012	0.29 ± 0.010
HLT (22-18ºC)	26.1 ± 1.88	25.2 ± 1.31[ab]	0.29 ± 0.019	0.30 ± 0.008

1088 Different letters indicate significant differences exist for calcium between fish with a different
1089 thermal history maintained at the same temperature. The results are shown as mean±s.e.m.; Two-
1090 way ANOVA; P<0.05.

1091