Thermal imprinting modifies bone homeostasis in cold challenged sea bream (*Sparus aurata*, L.)

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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.

Abstract

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

Recently considerable interest has been focused on determining the impact of thermal imprinting during embryonic and larval stages on the phenotypic plasticity of adult fish in part as a response to growing concern about the likely impact of global warming (Somero, 2005; Wood and McDonald, 1997). Thermal imprinting in early stages has a persistent effect on gene expression in subsequent stages (Garcia de la Serrana et al., 2012; Johnston et al., 2009; Jonsson and Jonsson, 2014; Scott and Johnston, 2012) and gene methylation and non-coding RNA have been suggested to contribute to the effect of temperature on developmental plasticity (Bizuayehu et al., 2015; Campos et al., 2014). Evidence has been gathered revealing that the thermal regime during early development can influence the juvenile stress response (Auperin and Geslin, 2008; Varsamos et al., 2006), muscle growth (Alami-Durante et al., 2007; Galloway et al., 1999; Garcia de la Serrana et al., 2012; Johnston et al., 2009; Macqueen et al., 2008; Steinbacher et al., 2011), and the incidence and character of skeletal deformities (Boglione and Costa, 2011). However, little is known about how embryonic or larval temperature regimes affect the ability of fish to cope with temperature changes in adult life. It is known that thermal imprinting in zebrafish (*Danio rerio)* embryos induce modified thermal tolerance in juveniles exposed to higher than normal culture temperatures (Schaefer and Ryan, 2006), but the effect of temperature during development on the physiological response to cold in adults is unstudied. In addition, the impact of early life temperatures on bone homeostasis have not previously been studied in adult teleost fish. Most of the studies that exist have looked at the effect of increased temperature on skeletal development in species such as tilapia (Campinho et al., 2004), European sea bass *Dicentrarachus labrax* (Koumoundouros et al., 2001), Atlantic salmon (Takle et al., 2005) and gilthead sea bream (Boglione and Costa, 2011) or how it affects the incidence of malformations, an issue of importance to aquaculture (Boglione et al., 2013; Koumoundouros, 2010).

Taking into consideration the role of temperature on thermal imprinting and subsequent performance of juveniles and adults and the known vulnerability of the skeleton to temperature induced changes in larvae (Divanach et al., 1996; Polo et al., 1991; Sfakianakis et al., 2011), we hypothesized that early thermal history from embryogenesis through the larvae-juvenile transition might influence the response of bone in adults to changes in water temperature characteristic of winter. To test this hypothesis adult fish with different thermal histories were exposed to a cold challenge typical of that experienced during winter. Since activation of the stress axis has previously been reported in winter syndrome (Rotllant et al., 2000), we assessed the response of adult fish with different thermal histories to a temperature drop by measuring plasma parameters associated with the stress response. The impact of thermal imprinting on bone metabolism during the temperature challenge was assessed by analysis of osteoblast and osteoclast activity by measuring the enzymatic activity of alkaline phosphatase (ALP, Dimai et al., 1998) and tartrate resistant acid phosphatase (TRAP, Persson et al., 1995), respectively, and determining the ash and calcium content of bone and the abundance of transcripts associated with the bone matrix. To assess if part of the effect of thermal imprinting occurred through modification of factors that regulate bone responsiveness, we analyzed the relative gene expression of regulatory factors

like insulin-like growth factor 1 (*igf1*), glucocorticoid receptor (*gr*) and thyroid receptors in bone (*trα* and *trβ*). Overall the objective of the study was to assess if thermal regime during sea bream development could influence the physiological response of young adults to a cold water challenge.

Material and Methods

Early life programming

All the procedures of early life temperature treatments and stress challenge were performed at the Institute for Aquaculture and Food Technology Research (IRTA), St. Carles de la Ràpita, Spain, in a temperature-controlled seawater recirculation system (IRTAmar™). All animal handling procedures were approved by the Ethics and Animal Care Committee (4998-T9900002) and complied with the guidelines of the European Union Council (86/609/EU), Spanish and Catalan Governments legislation.

Detailed information about the thermal imprinting experiments are provided in Garcia de la Serrana et al. (2012). In brief, fertilized eggs of gilthead sea bream (fertilization rate = 92%) were maintained at two different temperatures during embryogenesis, 18ºC (low temperature, LT) or 22ºC (high temperature, HT) in two independent temperature-controlled seawater recirculation systems. The two systems included two tanks of 2 m^3, and each contained two incubators (30 L) containing 110 mL of fertilized eggs. At hatching, larvae from replicate incubators within each temperature treatment were pooled, as no differences in hatching rate were observed, and they were then subdivided to generate the four different temperature regimes (2 replicate tanks/group, Fig. 1A). The temperature regimes were selected considering the two extreme temperatures of the optimal range for early life development of gilthead sea bream (18 and 22ºC) (Hough, 2010; Mozes et al., 2011): i) 18ºC from egg incubation through to hatching and up until larvae-juvenile transition (low temperature, LT); ii) 22ºC from egg incubation through to hatching and up until larvae-juvenile transition (high temperature, HT); iii) 18ºC from egg incubation up until hatching and then 22ºC until larvae-juvenile transition (low-high temperature, LHT); iv) 22ºC from egg incubation through to hatching and then 18ºC up until larvae-juvenile transition (high-low temperature, HLT). All treatment groups of juvenile fish were then maintained for seven months in duplicate 2 m^3 tanks per group, in a semi-closed recirculating sea water system with 5-10% water renewal/week, under a constant water temperature regime (21-22ºC). Juvenile fish were fed five times per day at 3% (kg/kg fish) with a commercial diet (OptiBream™).

A relatively large stock of thermally imprinted fish (adult fish in which the eggs and larvae were reared under different temperature regimes, approx. 700-900 per thermal regime) were generated and were used for several independent experiments (Garcia de la Serrana et al. (2012); Mateus et al., in press). Fish used for the present cold challenge experiment were age matched (7 months' post-hatch). Potential sex-related differences were not expected since the sea bream is a hermaphrodite and during

the first year mature as males (Pinto et al., 2006; Zohar et al., 1978). However, significant differences in weight and length existed between fish from the different thermal regimes (P<0.001; Table 1). The biometric differences detected in the present study between thermally imprinted fish were confirmed in a subsequent stress challenge experiment performed with 9 months post-hatch sea bream from the same stock of fish (Mateus et al., in press).

Cold challenge and sampling

To assess if thermal imprinting could modify the physiological response of young adult sea bream subjected to a cold water challenge, duplicate tanks of fish from each thermal regime (LT, LHT, HT and HLT) were randomly divided into two groups: the water temperature of the control groups was 23.0±1.0ºC and the cold challenge groups was 13.0±1.0ºC (Fig. 1B). Water temperature was progressively reduced at a rate of 1ºC per day, until the target temperature, 13ºC, was attained. Sea bream (n=10/group/ tank, see Table 1 for data on body weight, length, condition factor [K] and hepatosomatic index [HSI]) were exposed to reduced water temperature for 15 days. The circuit consisted of 200 L fiberglass tanks in a semi-closed sea water system at pH 7.5-8.0, 35-36‰ salinity and >80% oxygen saturation and maintained under a 12 h light/12 h dark photoperiod. Fish were fed to satiation and this corresponded to approximately 3% body weight daily using a commercial diet (OptiBream™) for the control groups and 1% body weight daily for the cold challenge since they would not eat more due to the cold stress. Uneaten food was siphoned daily from the bottom of the experimental tanks.

For sampling, fish were sacrificed with an overdose (450 ppm) of 2-phenoxyethanol (Sigma-Aldrich, USA), blotted dry and blood collected from the caudal vein using a heparinized syringe, centrifuged at 10,000 rpm for 4 minutes at 4ºC, and the plasma stored at -20ºC. Haemal vertebrae (bone) were collected into RNA later for subsequent RNA extraction, enzymatic assays and calcium and mineral content analysis. Vertebrae samples were incubated overnight at 4ºC in RNA later and then stored at -80ºC until analyses.

Plasma analyses

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

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

Bone TRAP and ALP activities

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

Calcium and ash content in bone

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

0.5 and 10 ppm (parts per million) of calcium (Agilent Calibration Mix Majors 6610030700). Running parameters for MP-AES were pump rate 15 rpm, sample uptake time 70 sec, rinse time 40 sec, stabilization time 15 sec, with 5 replicate readings and the selected options "fast pump during uptake" and "rinse time fast pump" in mode "on". Calcium contents were measured at a wavelength of 393 nm and then expressed as µmol.mg^{-1}.

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

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

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

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

Statistical analysis

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

Results

Biometric parameters

Two-way ANOVA revealed that body weight and length of adult fish were affected by thermal history ($P<0.001$), whereas condition factor K was affected by cold temperature challenge ($P<0.01$, Table 1). HSI was affected by both thermal history and the temperature challenge ($P<0.001$) and also by the interaction between both factors ($P<0.01$).

Fish from LHT group were significantly heavier and larger ($P<0.001$) than fish from other thermal groups irrespective of water temperature. However, no significant covariation was detected between body mass and the other physiological parameters monitored. Fish exposed to a cold temperature challenge (13±1ºC) for 15 days had a significantly higher HSI ($P<0.01$, LT and LHT;

P<0.001, HLT and HT) than those maintained at 23±1ºC irrespective of their thermal history. The condition factor K did not differ significantly between fish with the same thermal history maintained at 23±1ºC or exposed to 13±1ºC. The exception was the LT fish in which K was significantly (P<0.05) lower in the cold challenged (13±1ºC) group relative to the fish maintained at 23±1ºC. No significant differences were found by the end of the experiment in the weight or length of fish from the same thermal history maintained at 23ºC or exposed to 13ºC for 15 days.

Characterization of the physiological response to cold stress

Plasma cortisol

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

Plasma glucose

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

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

Na^+ and K^+ plasma concentrations were significantly (P<0.05 and P<0.01, respectively) affected by thermal history and the cold temperature challenge, whereas protein and osmolality were only significantly (P<0.001 and P<0.05, respectively) affected by a cold challenge (Table 3). Plasma Na^+ was also significantly (P<0.001) affected by the interaction between both factors. Plasma Na^+, K^+, protein and osmolality in LT, LHT, HT and HLT fish maintained at 23±1ºC were not significantly

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

Characterization of bone metabolism in response to cold stress

TRAP and ALP activity in vertebrae

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

Calcium content in vertebrae

Two-way ANOVA revealed a significant (P=0.02) effect of thermal history on the calcium content of haemal vertebrae (Table 4). Comparison of the calcium content in the vertebrae of the LT, LHT, HT and HLT fish maintained at 23±1ºC revealed no significant differences between groups. Comparison of LT, LHT, HT and HLT fish exposed to a cold challenge (13±1ºC) revealed that the LHT fish had a significantly (P=0.03) higher calcium content than fish of the LT regime. No significant differences in ash content of vertebrae were detected in fish with the same thermal history that were maintained at 23±1ºC or exposed to 13±1ºC for 15 days (Table 4).

Gene expression in bone in response to cold stress

Transcripts of the bone matrix in vertebrae

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

Transcripts of regulatory factors in vertebrae

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

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

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

Discussion

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

Thermal challenge and somatic indexes

In line with previous reports, a decrease in water temperature was associated with a significant reduction in feed intake, which is one of the first signs of cold stress (Tort et al., 2004). However, in the present study irrespective of thermal history, the reduction in feed intake as a consequence of a drop in water temperature did not affect body weight or K, which was similar to the matched controls maintained at 23±1ºC. These results are in line with other studies of cold challenged sea bream, in which body weight was not affected by low water temperatures (Tort et al., 2004), although HSI was increased as a consequence of the failure to mobilize fat stores (Ibarz et al., 2007; Ibarz et al., 2005). We propose that the maintenance of body weight and K in the present study indicates that sea bream were able to adjust their metabolism to compensate for the effects of a short-term (15 days) cold challenge as it has been shown for other fish (Hochachka and Somero, 1984). Our results contrast with previous studies in which sea bream were unable to maintain their body mass presumably because the water temperature in previous studies was dropped to below 10ºC and the fish totally stopped feeding (Ibarz et al., 2003). Overall, our results suggest that thermal imprinting did not influence the capacity of the sea bream to compensate their metabolism when water temperature was reduced.

Thermal challenge as a stressor

In the present study, the cortisol response at different time points during the experiment was not established and so it was not possible to confirm if a drop in water temperature caused a transient peak in cortisol as previously reported in the gilthead sea bream (Rotllant et al., 2000) and the Atlantic cod (Gadus morhua, Staurnes et al., 1994). Furthermore, increased plasma glucose (a secondary stress marker, Pottinger and Pickering, 1997) was only observed in the HLT group when fish were exposed to 13ºC for 15 days. However, the results from several previous studies suggest that the development of hyperglycemia in response to a cold challenge is variable in this species (Sala-Rabanal et al., 2003; Tort et al., 2004; Vargas- Chacoff et al., 2009). Notably, the only group that was hyperglycemic in our experiments (the HLT group) was also the group that had significantly higher plasma cortisol (108.4±71.4 ng.mL^{-1}). A positive correlation between plasma cortisol and glucose has been previously reported in Atlantic cod under cold stress (Staurnes et al., 1994). By the end of the cold challenge, a drastic reduction in plasma cortisol occurred in the LT, LHT and HT groups relative to the same thermal group maintained at 23ºC, which is in agreement with the results of previous studies in the gilthead and silver sea bream (Deane and Woo, 2005; Rotllant et al., 2000). The results of the present study indicate that in the gilthead sea bream thermal imprinting modified the cortisol response in adults when they were exposed to a cold challenge, presumably through modifications in the stress axis. In fact, in a previous study, exposure to an acute stress challenge of slightly older fish (9 months old) from the same population of fish revealed that thermal imprinting caused significant changes in the central stress axis (Mateus et al., in press).

The reference resting values for plasma cortisol in gilthead sea bream are between 1-10 ng.mL^{-1} and for chronic (around 33±34.1 ng.mL^{-1}) and acute stress (162±101.8 ng.mL^{-1}) (Tort, *et al*, 2011) are significantly higher. Surprisingly, plasma cortisol levels in fish maintained at 23ºC under standard experimental conditions were those characteristic of a stress response. The elevated cortisol levels may have been a result of the acute stress of capture and handling (Laidley and Leatherland, 1988; Molinero et al., 1997) even though we endeavored to minimize stress during sampling. The results tend to suggest that the stress response in the 13ºC challenged LT, LHT and HT groups was suppressed, although the mechanism by which this occurred was not established in the present study and will be a target for future studies.

The present study confirmed the hypothesis raised by others (Beitinger et al., 2000; Somero, 2005) that thermal history influences thermal tolerance in adult fish. To our knowledge, only one other study has investigated the effects of thermal history on the thermal tolerance of adult fish and it involved exposing zebrafish to high water temperatures (Schaefer and Ryan, 2006), but did not assess how the challenge modified physiological and endocrine systems. The results of our study confirm the general notion that non-lethal stress in early life may modify whole animal physiology and favor improved acclimation to stressors in later life (Jones, 2012). However, the results of our study indicate that the

characteristics and timing of the stress, in this case temperature, may play a crucial role in determining the impact on adult physiology. For example, the physiological response of the LHT and HLT groups of gilthead sea bream to a low temperature challenge differed. At the end of the cold challenge, the HLT fish had higher glucose and cortisol levels, while the LHT fish had a suppressed cortisol response that reached the resting levels and plasma glucose levels were unchanged, which may suggest that LHT fish were more apt at acclimating to a low water temperature. This supports the notion that embryonic stage may be a critical window of increased susceptibility to temperature induced changes in fish development (Scott and Johnston, 2012; Skjærven et al., 2011).

Thermal challenge and plasma parameters

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

A drop in water temperature has previously been reported to produce an imbalance in plasma chemistry, which can impact on a number of processes including metabolism and osmoregulation (Donaldson et al., 2008; Ibarz et al., 2010b; Rotllant et al., 2000). Previous studies have revealed that cold water challenge in gilthead sea bream caused an imbalance in plasma ions and most notably a reduction in plasma calcium, sodium and potassium levels (Gallardo et al., 2003; Rotllant et al., 2000; Sala-Rabanal et al., 2003; Vargas- Chacoff et al., 2009) and a significant increase in osmolality in juvenile turbot (*Scopthalmus maximus*, Imsland et al., 2003) and in tilapia hybrids (*Oreochromis mossambicus* x *O. urolepis hornorum*, Sardella et al., 2004). The modified plasma ion profile in cold challenged sea bream has been linked to a change in their osmoregulatory capacity, resulting from a change in the morphology of the gill epithelium and a drastic reduction in gill, intestine and kidney Na^+/K^+-ATPase activity (Ibarz et al., 2010b). Overall, the results for plasma chemistry in the present study suggest that thermal imprinting had differing consequences for the osmoregulatory response to cold challenge in the gilthead sea bream. In particular, the thermal regimes associated with least change in plasma chemistry in response to a cold challenge was the LT and LHT group, while in contrast, the HLT group suffered a significant reduction in plasma sodium and potassium levels and a significant increase in osmolality relative to the matched group maintained at 23ºC. The mechanism by which thermal imprinting modified plasma chemistry was not established in the present study, but may result from the changes induced by temperature in the developmental events occurring during embryo and early larval development (Yúfera et al., 2011).

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

Thermal challenge and bone homeostasis

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

The vertebral bone in gilthead sea bream from different thermal histories had a different response to a cold challenge and the enzymatic activities of TRAP and ALP, and ECM and hormone receptor transcript abundance were modified. The reduction in temperature associated with cold challenge caused a simultaneous reduction in ALP and TRAP enzyme activity in fish of LT and HLT groups. However, only fish from the LT group also had a decrease in bone calcium content and a significant down-regulation of *ogn1*, which in other studies has been shown to be indicative of modified bone remodeling in fish (Pombinho et al., 2004) and rat (Goto and Tsukamoto, 2003). Although in the LHT group exposed to 13ºC ALP and TRAP were not significantly modified relative to the matched group at 23ºC, *ocn* and *ogn1/2* were significantly down-regulated, which is in line with the results of

previous studies on fasted sea bream (Vieira et al., 2013) and type I diabetic mice (Botolin et al., 2005). These results may suggest that later stages of osteoblast differentiation were suppressed, while earlier stages were unaffected. If the changes observed in bone from fish with different thermal histories, arose from epigenetic mechanisms was not established in this study. However, evidences exist that temperature during early development causes epigenetic modulation in the genome in teleosts (Bizuayehu et al., 2015; Campos et al., 2014). Furthermore, in Atlantic cod reared at different temperatures after hatching the expression of miRNAs associated with bone activity was modified (Bizuayehu et al., 2015), and suggests a possible mechanism by which early rearing temperature can influence adult bone.

Bone is an emerging endocrine tissue (Blair et al., 2008) and also a target for a number of endocrine hormones, such as glucocorticoids, thyroid hormone and insulin like growth factor that regulate its turnover (Robson et al., 2002). The effect of a cold challenge on the responsiveness of bony tissue in ectotherms and particularly those with different early thermal histories has never been studied. Candidate transcript abundance was similar in all experimental groups at 23ºC, suggesting thermal imprinting did not appear to modify basal bone metabolism in adult sea bream. However, thermal imprinting changed the response of bone to a drop in water temperature and *igf1*, associated with growth and bone turnover (Collins et al., 1998; Gabillard et al., 2005; Ono et al., 1996), *trα* and *trβ*, associated with bone resorption (Blair et al., 2008; Sbaihi et al., 2007) and *gr* that mediates the effects of cortisol (Moutsatsou et al., 2012), were all significantly down-regulated in the LHT group vertebral bone. These results suggest that a drop in water temperature impairs the responsiveness of bone by repressing the transcription of these genes (Abbas et al., 2012; Larsen et al., 2001) and that this in turn impairs bone remodeling (Suzuki and Hattori, 2002). It would be of interest to directly measure the change in bone ECM proteins to assess the impact of thermal history and cold challenge on vertebral bone mass, but since neither antisera or assays are currently available for fish, this was not possible. Nonetheless, an intriguing observation was that the groups with the most significant down-regulation of bone matrix transcripts (LHT, LT and HT) also had the most notable down-regulation of *gr*, *igf1*, *trα* and *trβ*. Although a simultaneous decrease in TRAP and ALP activity was detected in HLT group, no modification was identified in bone calcium content and ECM transcripts relative abundance which may be justified by an unchanged endocrine response in the bone of fish of the HLT group. This observation is in line with previous studies which have revealed that disruption of endocrine signaling including thyroid (Sbaihi et al., 2007; Takagi et al., 1994) and cortisol in fish (Sbaihi et al., 2009) and mice (Sher et al., 2006) modifies bone cell responsiveness to regulatory factors. Overall, although thermal imprinting failed to modify bone metabolism and responsiveness in optimal ambient water temperatures, it did modify the response of bone to a cold challenge. Future studies should be directed at establishing the epigenetic mechanisms underlying this response.

List of symbols and abbreviations

GR, glucocorticoid receptor;
HLT, high-low temperature;
HSI, hepatosomatic index;
HT, high temperature;
IGF1, insulin-like growth factor 1;
K, condition factor;
LHT, low-high temperature;
LT, low temperature;
OCN, osteocalcin;
OGN, mimecan/osteoglycin.
pNP, para-nitrophenol;
pNPP, para-nitrophenyl phosphate;
TRα/β, thyroid receptors α or β;

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Competing interests

The authors declare no competing or financial interests.

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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Figures

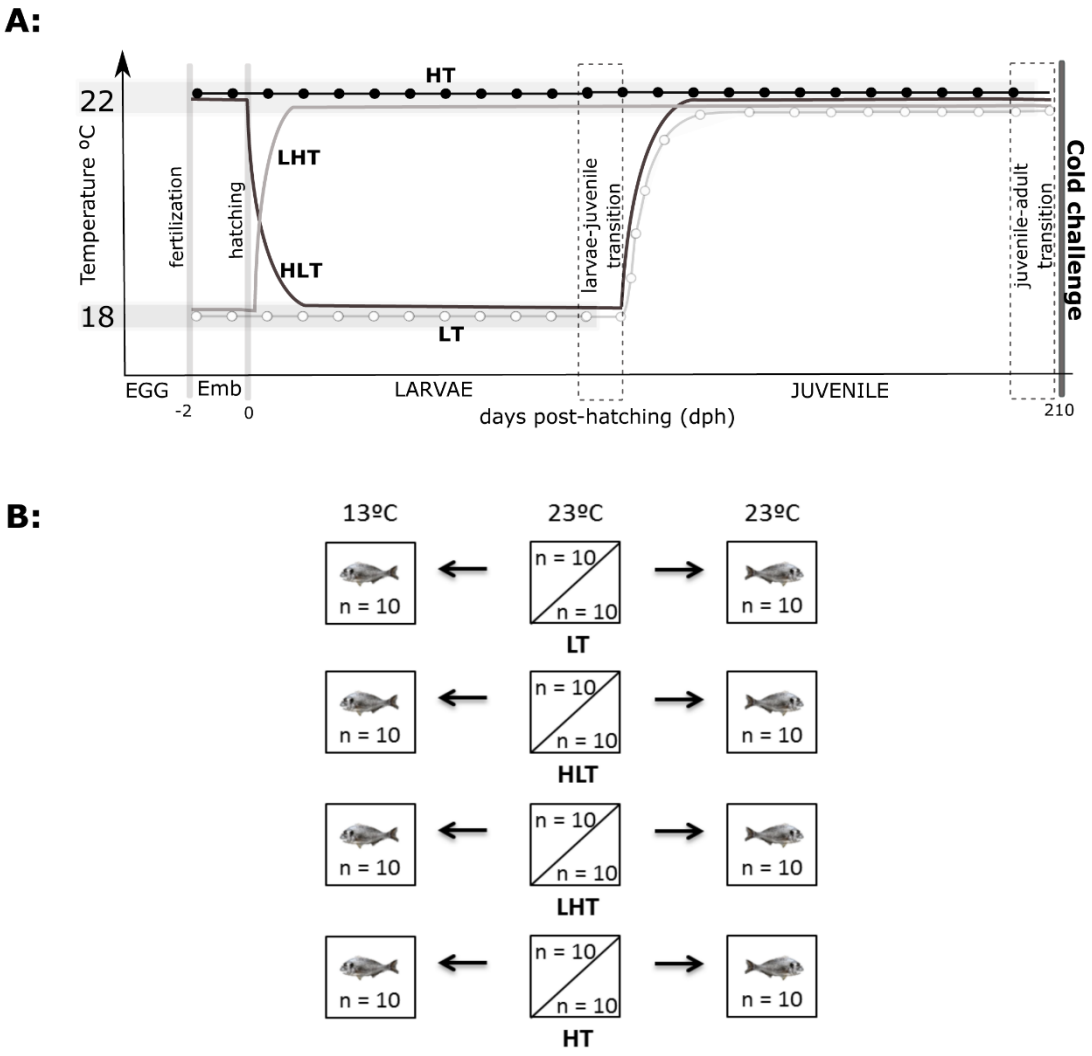


Fig. 1. Schematic representation of the temperature regimes that gilthead sea bream were exposed from egg fertilization to larvae-juvenile transition until the cold challenge. A: Four temperature treatments (thermal groups) were generated, two with constant temperatures (LT, low temperature [18-18ºC] and high temperature, HT [22-22ºC]) and two with variable temperatures during the egg incubation phase and larval rearing (HLT [22-18ºC] and LHT [18-22ºC]). Fish from all thermal groups were maintained at a common temperature (22±1ºC) from the larvae-juvenile transition (when the body was covered with scales) for 7 months until the beginning of the cold challenge. B: The cold challenge was performed by randomly dividing each group of fish for a thermal regime into two groups. The control group was maintained in replicate tanks at 23ºC and

the cold group was maintained in replicate tanks at 13ºC (n=10/thermal history group). Fish were subjected to these temperatures for 15 days until sampling.

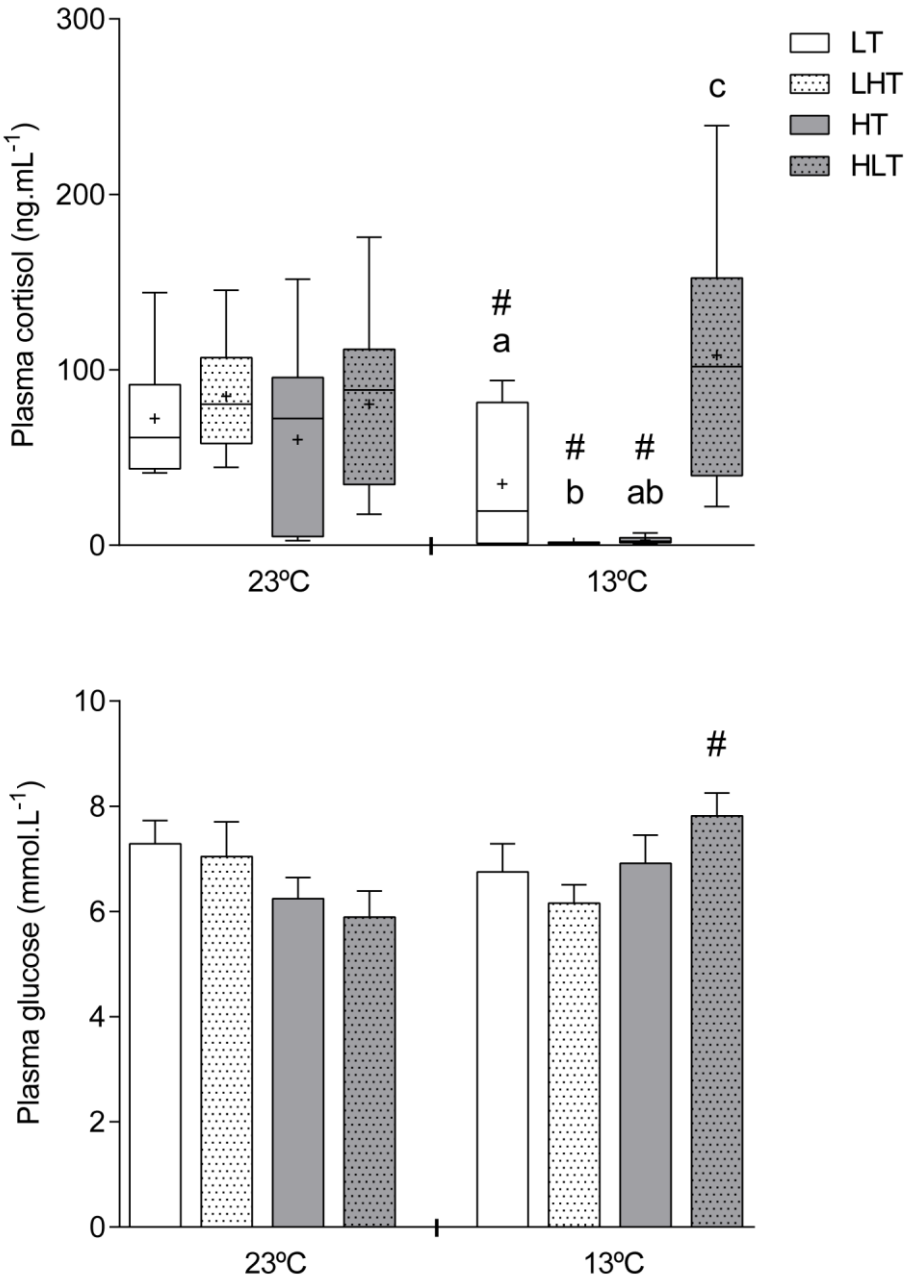


Fig. 2. Cortisol and glucose plasma levels. The stress related parameters were analyzed in plasma samples from sea bream maintained in replicate tanks under control conditions (23ºC, n=10/thermal history) or under a cold challenge (13ºC, n=10/thermal history group) for 15 days. The cortisol levels are plotted in a Tukey box plot and whiskers graph (with '+' representing the mean) and the results of glucose are shown as mean±s.e.m. of the groups with different thermal history: LT (18-18ºC); LHT (18-22ºC); HT (22-22ºC); HLT (22-18ºC). Different letters indicate significant differences exist for cortisol levels between fish with a different thermal history maintained at the same temperature. Cardinal (#) indicates significant differences exist on glucose levels between fish with the same thermal history maintained at different temperatures, 23ºC or 13ºC. Two-way ANOVA; $P<0.05$.

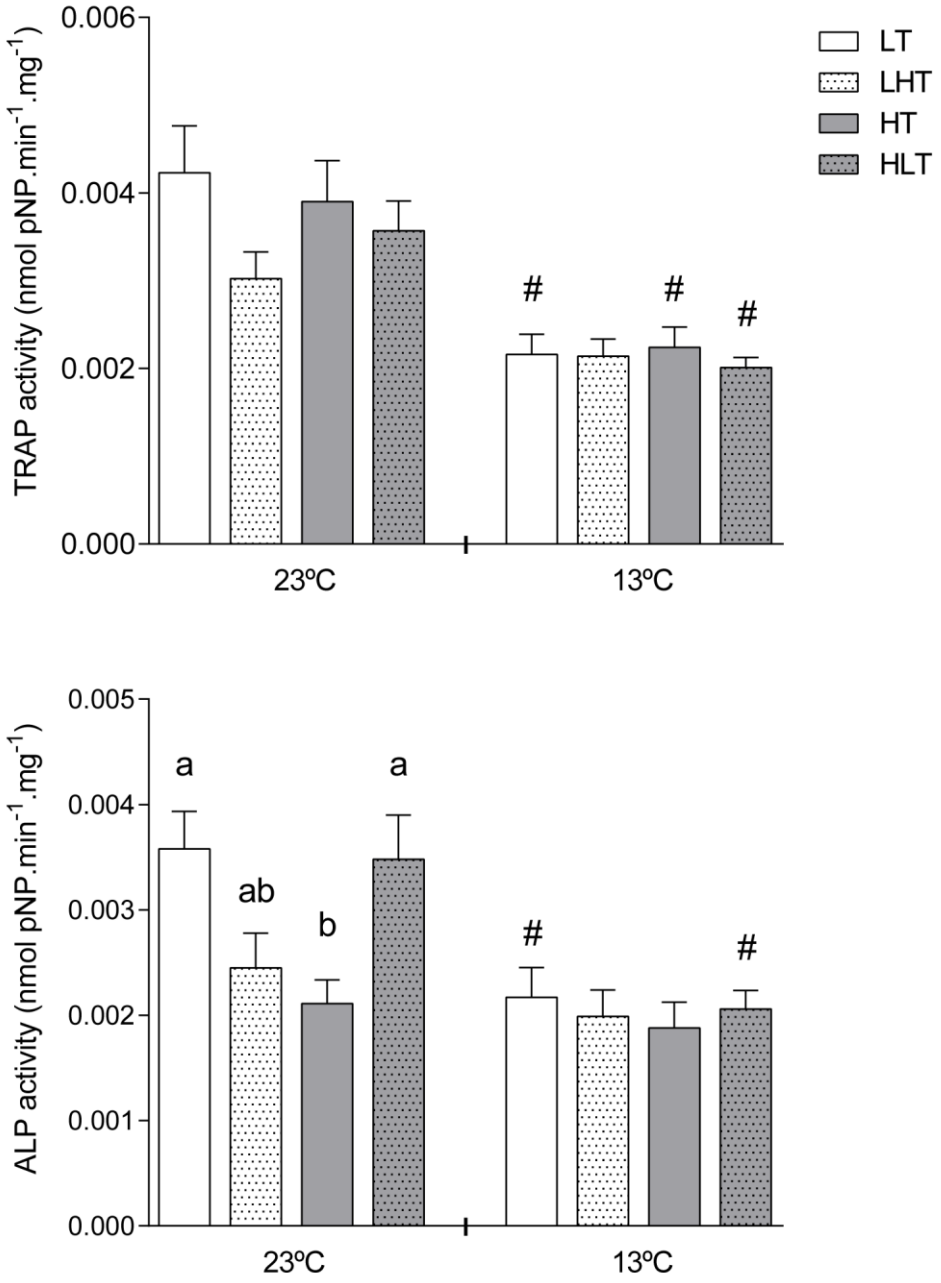
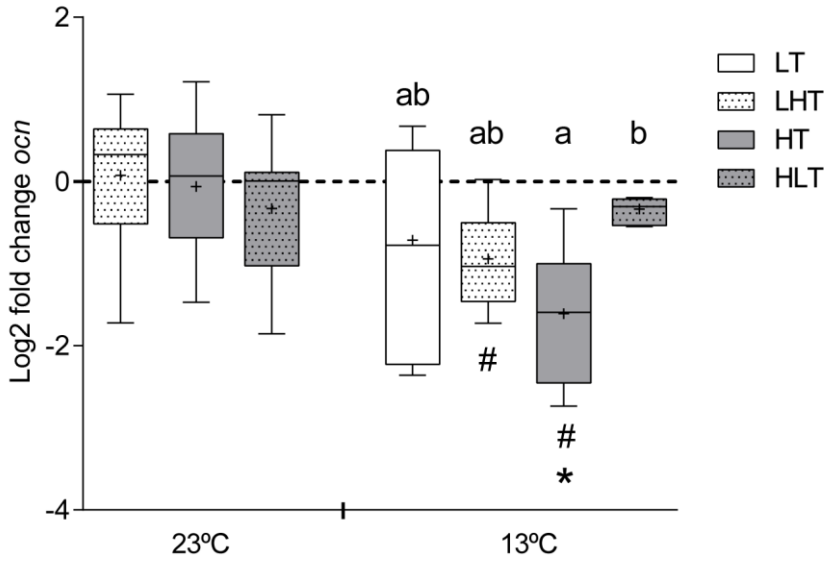
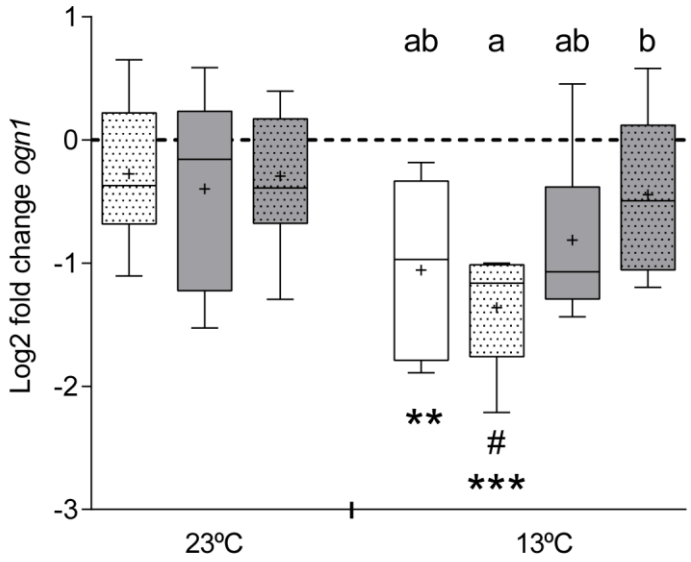


Fig. 3. Effects of low temperature challenge on biochemical markers of bone remodeling ALP and TRAP, measured in vertebral bone of sea bream with different thermal histories in the control (23ºC, n=10/thermal history) and cold challenged group (13ºC, n=10/thermal history group) 15 days after acclimation to the temperatures. The results of the replicate tanks/ treatment were pooled for statistical analysis as no significant differences were found. The results obtained for fish from each thermal regime, LT (18-18 ºC); LHT (18-22 ºC); HT (22-22 ºC); HLT (22-18 ºC) are represented. Different letters indicate significant differences exist for ALP activity between fish with a different thermal history maintained at the same temperature. Cardinal (#) indicates significant differences existed between fish with the same thermal history maintained under control conditions 23ºC or exposed to a cold challenged, 13ºC. The results are shown as

mean±s.e.m. of para-nitrophenol (pNP) production (nmol pNP.min.mg^{-1}). Statistical significances (by Two-way ANOVA) were set at P<0.05.





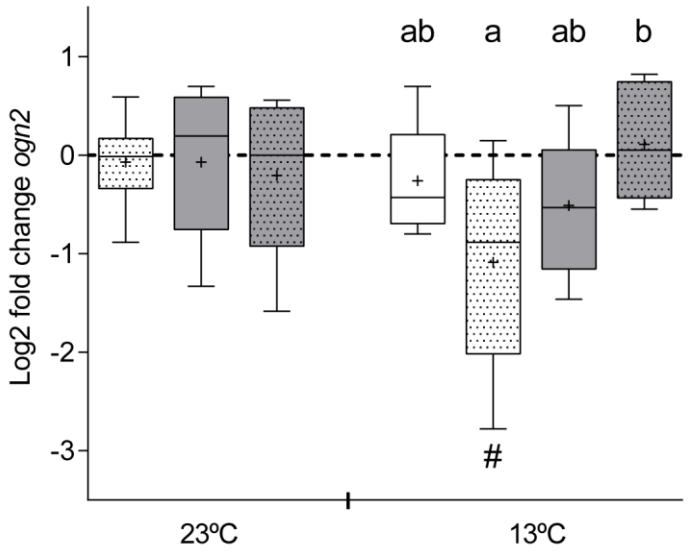


Fig. 4. Relative expression of transcripts associated with the bone matrix: *ocn*, *ogn1* and *ogn2*. Vertebral bone cDNA for each individual was analyzed by qPCR and normalized by the mean of *18s* expression: control group (23ºC, n=10/thermal history) and cold group (13ºC, n=10/thermal history group).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

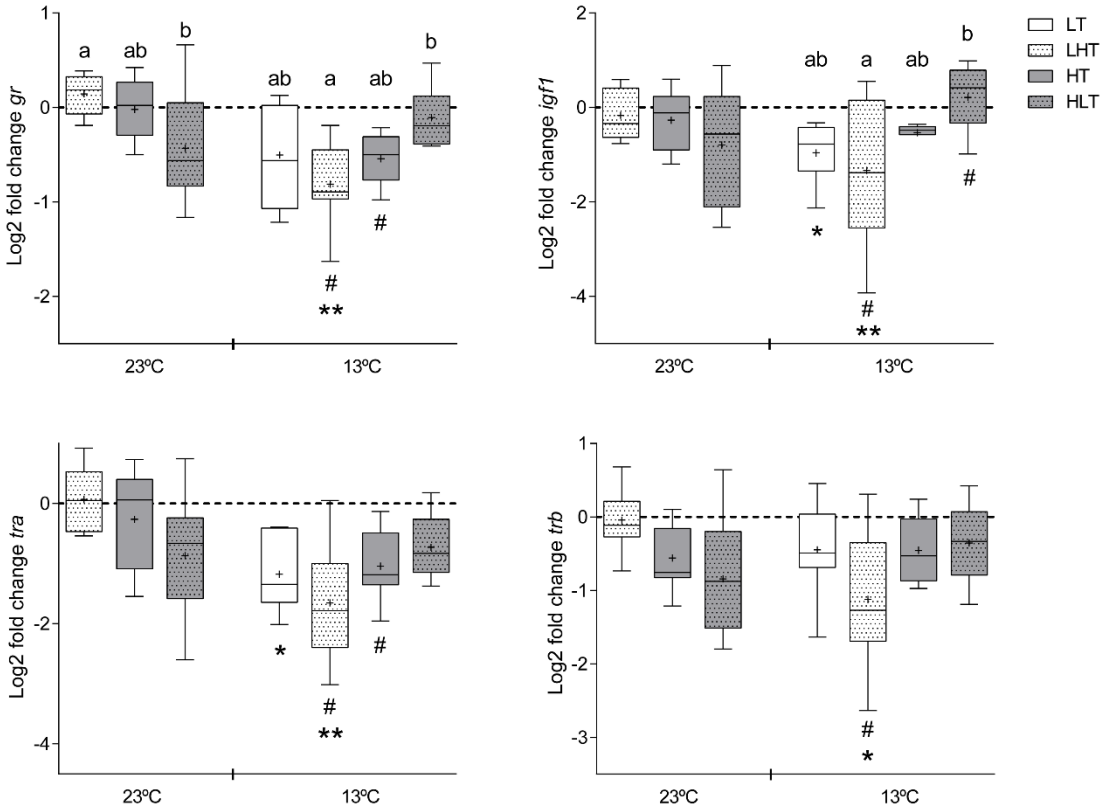


Fig. 5. Relative expression of transcripts associated with endocrine responsiveness at low temperatures: *gr*, *igf1*, *trα* and *trβ*. Vertebral bone cDNA for each individual was analyzed by qPCR and normalized by the mean of *18s* expression: control group (23ºC, n=10/thermal history) and cold group (13ºC, n=10/thermal history group). Results for each thermal history group are expressed as Log2 Fold change relative to the LT group (thermal history 18-18ºC) maintained at 23ºC, defined as control and not represented (corresponds to the base line with fold change=0); results are represented in a Tukey box plot: LT (18-18ºC); LHT (18-22ºC); HT (22-22ºC); HLT (22-18ºC). '+' represents the mean. The results of the replicate tanks/treatment were pooled for statistical analysis as no significant differences were found between them. Different letters indicate significant differences between the thermal groups maintained at the same temperature. Cardinal (#) indicates significant differences occurring between fish with the same thermal history maintained under control conditions 23ºC or exposed to a cold challenge, 13ºC. Significant up-regulation or down-regulation relative to the control (taken at the LT group maintained at 23ºC) was denoted by: * $P<0.05$, ** $P<0.01$ using Two-Way ANOVA.

Tables

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

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

Table 2: Primers used for gene expression analysis by quantitative RT-PCR. Gene name, accession number, primer sequence, amplicon length (bp), annealing temperature (T, ºC) and 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

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

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

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

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

Supplementary Tables

Table S1 – Tukey's post hoc test for analysis of significant differences across all groups in biometric parameters. Groups that are significantly different for specific parameters are identified by different letters. $P < 0.05$; n=10/group.

Water Temperature	23ºC				13ºC			
Thermal History	LT	LHT	HT	HLT	LT	LHT	HT	HLT
Body Weight	a	bc	b	a	a	c	d	a
Standard Length	a	bc	bd	ae	a	c	de	ade
HSI	ab	ab	ab	b	cd	ad	c	cd
K	ns	ns	ns	ns	ns	ns	ns	ns

ns – not significantly different

Table S2 - Tukey's post hoc test for analysis of significant differences across all groups in biochemical parameters. Groups that are significantly different for specific parameters are identified by different letters. $P < 0.05$; n=10/group.

Water Temperature	23ºC				13ºC			
Thermal History	LT	LHT	HT	HLT	LT	LHT	HT	HLT
Cortisol	a	a	ab	a	a	c	c	bc
Glucose	ns	ns	ns	ns	ns	ns	ns	ns
Sodium	a	a	a	a	a	a	a	b
Potassium	a	ab	ab	ab	ab	b	ab	b
Protein	a	ab	a	a	ab	ab	b	ab
Osmolality	ns	ns	ns	ns	ns	ns	ns	ns

ns – not significantly different

Table S3 - Tukey's post hoc test for analysis of significant differences across all groups in bone parameters. Significant differences were identified by using different letters. $P < 0.05$; n=10/group.

Water Temperature	23ºC				13ºC			
Thermal History	LT	LHT	HT	HLT	LT	LHT	HT	HLT
TRAP	a	abc	a	ac	bc	bc	bc	b
ALP	a	ab	b	a	b	b	b	b
Calcium	ns	ns	ns	ns	ns	ns	ns	ns
Ash	ns	ns	ns	ns	ns	ns	ns	ns

ns – not significantly different

Table S4 – Tukey's post hoc test for analysis of significant differences across all groups in relative abundance of bone transcripts. Significant differences were identified by using

Water Temperature	23ºC				13ºC			
Thermal History	LT	LHT	HT	HLT	LT	LHT	HT	HLT
ocn	ab	a	a	ab	ab	ab	b	ab
ogn1	a	a	ab	a	ab	b	ab	ab
ogn2	ab	ab	ab	ab	ab	a	ab	b
gr	ab	a	ab	abc	abc	c	bc	ab
igf1	a	ab	ab	ab	ab	b	ab	a
trα	ab	a	ab	abc	bc	c	abc	abc
trβ	ab	a	ab	ab	ab	b	ab	ab

different letters. $P < 0.05$; n=10/group.

Supplementary Tables

Table S1 – Tukey's post hoc test for analysis of significant differences across all groups in biometric parameters. Groups that are significantly different for specific parameters are identified by different letters. $P < 0.05$; n=10/group.

Water Temperature	23ºC				13ºC			
Thermal History	LT	LHT	HT	HLT	LT	LHT	HT	HLT
Body Weight	a	bc	b	a	a	c	d	a
Standard Length	a	bc	bd	ae	a	c	de	ade
HSI	ab	ab	ab	b	cd	ad	c	cd
K	ns	ns	ns	ns	ns	ns	ns	ns

ns – not significantly different

Table S2 - Tukey's post hoc test for analysis of significant differences across all groups in biochemical parameters. Groups that are significantly different for specific parameters are identified by different letters. $P < 0.05$; n=10/group.

Water Temperature	23ºC				13ºC			
Thermal History	LT	LHT	HT	HLT	LT	LHT	HT	HLT
Cortisol	a	a	ab	a	a	c	c	bc
Glucose	ns	ns	ns	ns	ns	ns	ns	ns
Sodium	a	a	a	a	a	a	a	b
Potassium	a	ab	ab	ab	ab	b	ab	b
Protein	a	ab	a	a	ab	ab	b	ab
Osmolality	ns	ns	ns	ns	ns	ns	ns	ns

ns – not significantly different

Table S3 - Tukey's post hoc test for analysis of significant differences across all groups in bone parameters. Significant differences were identified by using different letters. $P < 0.05$; n=10/group.

Water Temperature	23ºC				13ºC			
Thermal History	LT	LHT	HT	HLT	LT	LHT	HT	HLT
TRAP	a	abc	a	ac	bc	bc	bc	b
ALP	a	ab	b	a	b	b	b	b
Calcium	ns	ns	ns	ns	ns	ns	ns	ns
Ash	ns	ns	ns	ns	ns	ns	ns	ns

ns – not significantly different

Table S4 – Tukey's post hoc test for analysis of significant differences across all groups in relative abundance of bone transcripts. Significant differences were identified by using

Water Temperature	23ºC				13ºC			
Thermal History	LT	LHT	HT	HLT	LT	LHT	HT	HLT
ocn	ab	a	a	ab	ab	ab	b	ab
ogn1	a	a	ab	a	ab	b	ab	ab
ogn2	ab	ab	ab	ab	ab	a	ab	b
gr	ab	a	ab	abc	abc	c	bc	ab
igf1	a	ab	ab	ab	ab	b	ab	a
trα	ab	a	ab	abc	bc	c	abc	abc
trβ	ab	a	ab	ab	ab	b	ab	ab

different letters. $P < 0.05$; n=10/group.