

1 **Cimetidine disrupts the renewal of testicular cells and the steroidogenesis in a**  
2 **hermaphrodite fish.**

3 **Short title:** Cimetidine disrupts the gonad of gilthead seabream

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22 **Footnote<sup>1</sup>**: The genetic nomenclature used in this manuscript follow the guidelines of  
23 Zebrafish Nomenclature Committee (ZNC) for fish genes and proteins and the HUGO  
24 Gene Nomenclature committee for mammalian genes and proteins.

25 Footnote<sup>2</sup>: 11-ketotestosterone (11KT), 17 $\beta$ -estradiol (E<sub>2</sub>), 4% paraformaldehyde in PBS  
26 (PAF), 5-bromo-2'-deoxyuridine (BrdU), aromatase gene (*cyp19a1a*), body mass (BM),  
27 chlorpheniramine (Chr), cholesterol side chain cleavage cytochrome P450 gene (*cyp11a1*),  
28 cimetidine (Cim), days post-injection (dpi), dimaprit (Dm), double sex-and mab3-related  
29 transcription factor 1 gene (*dmrt1*), famotidine (Fam), fetal bovine serum (FBS), gonad  
30 mass GM), gonadosomatic index (GSI), histamine receptors (HRs), *in situ* detection of  
31 DNA fragmentation (TUNEL), intraperitoneally (ip), monoclonal antibody (mAb), mAb  
32 specific to gilthead seabream acidophilic granulocytes (G7), phorbol myristate acetate  
33 (PMA), polyclonal antibody (pAb), pyridilethylamine (Peth), ribosomal protein S18 gene  
34 (*rps18*), steroid 11-beta-hydroxylase gene (*cyp11b1*), steroidogenic acute regulatory protein  
35 gene (*star*).

36

37

38 **Abstract**

39 The importance of histamine in the physiology of the testis in mammals and reptiles has  
40 been recently shown. Histamine receptors (Hrs) are well conserved in fish and are  
41 functional in several fish species. We report here for the first time that histamine and the  
42 mRNA of Hrh1, Hrh2 and Hrh3 are all present in the gonad of the hermaphrodite teleost  
43 fish gilthead seabream. Moreover, cimetidine, which acts *in vitro* as an agonist of Hrh1 and  
44 Hrh2 on this species, was intraperitoneally injected in one and two years old gilthead  
45 seabream males. After three and five days of cimetidine injection, we found that this  
46 compound differently modified the gonadal *hrs* transcript levels and affects the testicular  
47 cell renewal and the gene expression of steroidogenesis-related molecules as well as the  
48 serum steroid levels. Our data point to cimetidine as a reproductive disruptor and elucidate  
49 a role for histamine in the gonad of this hermaphrodite fish species through Hr signalling.

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51 **Keywords:** Cimetidine, histamine receptors, hermaphrodite fish, testis.

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## 54 **1. Introduction**

55 Cimetidine (Cim) is a pharmacological compound found in surface watercourses and  
56 recently in a coastal marine lagoon (Mar Menor, Spain) (Kolpin et al., 2002; Moreno-  
57 González et al., 2014; Ternes et al., 2001), Although no information exists on the presence  
58 of Cim in the open marine environment, it could be an emerging contaminant, as  
59 pharmaceuticals are consumed all over the world, mainly in seas with little water exchange  
60 such as the Mediterranean Sea or nearby to the coast where fish farming occurred. In that  
61 sense, the assessment of how Cim can influence or disrupt marine living species physiology  
62 is important to define effective constrains measures. Measures that becomes highly  
63 important for commercial species with an important economic value for aquaculture such as  
64 the marine gilthead seabream (*Sparus aurata* L.). Cim has been reported in mammals as a  
65 potent antagonist of histamine H<sub>2</sub>-receptor (HRH<sub>2</sub>), but also as a partial agonist or as an  
66 inverse agonist of HRH<sub>2</sub> depending on the ability of the cell to regulate the amount of  
67 HRH<sub>2</sub> upon long term exposure to Cim (Smit et al., 1996; Takahashi et al., 2006; van der  
68 Goot and Timmerman, 2000). In mammals, Cim enhances tumor infiltrating lymphocytes  
69 responses, the antigen presenting capacity of dendritic cells and the interleukin 18 (IL18)  
70 production of monocytes, showing a potent anti-oxidative activity while also reducing pro-  
71 inflammatory cytokines production (Kubecova et al. 2011; Kubota et al., 2002; Takahashi  
72 et al., 2006). Moreover, Cim triggered the apoptosis of several testicular cell types and has  
73 been reported as a reproductive toxicant in male rats (Franca et al., 2000; Sasso-Cerri and  
74 Cerri, 2008). Recently, it has been reported that the dietary intake of Cim alters the non  
75 specific immunity in carps (Hosseini et al., 2013). Moreover, the exposure of adult

76 zebrafish to environmental concentrations range from 3 to 300 mg/L of Cim alters several  
77 reproductive parameters (Lee et al., 2015).

78 Histamine receptors (Hrs) are well conserved in fish (60% amino acid similarity  
79 between fish and mammals). Thus, homologues for Hrh1, Hrh2 and Hrh3 have been  
80 identified in the zebrafish and shown to be functional (Peitsaro et al., 2007). Moreover,  
81 recent studies have shown that histamine is present in fish belonging to the Perciformes  
82 order, the largest and most evolutionarily advanced order of teleosts. Concretely, the  
83 gilthead seabream (*Sparus aurata* L.), a hermaphrodite species, shows eosinophilic  
84 granules cells that differ from acidophilic granulocytes (the professional phagocytes of  
85 gilthead seabream) in the presence of histamine stored in their granules (Mulero et al.,  
86 2007). Moreover, in the gilthead seabream, histamine is biologically active and is able to  
87 regulate the inflammatory response by acting on professional phagocytes (Mulero et al.,  
88 2007).

89 Histamine has also a relevant role in the physiology of the testis in mammals  
90 (Mayerhofer et al., 1989) and reptiles (Khan and Rai, 2007; Minucci et al., 1995). In fact, in  
91 mammals, histamine has been reported to regulate Leydig cell physiology through HRH1  
92 and HRH2 (Albrecht et al., 2005; Mondillo et al., 2005; Pap et al., 2002). Although  
93 macrophages, granulocytes and lymphocytes have been described in the testis of teleosts  
94 (Cabas et al., 2011; Chaves-Pozo et al., 2003; Liarte et al., 2007; Lo Nostro et al., 2004),  
95 which are physiologically involved in the development of some reproductive stages  
96 (Chaves-Pozo et al., 2005a, b; Liarte et al., 2007), nothing is known about the presence  
97 and/or ability of histamine to influence fish gonad physiology or steroidogenesis.

98           As a protandrous hermaphrodite teleost, the gilthead seabream develops as male  
99 during the first 2-3 years depending on different environmental conditions. Although  
100 testicular area is quickly differentiated and spermatozoa are produced in less than one year  
101 old fish, those fish are not spermiogenic active males (Chaves-Pozo et al., 2009). As a  
102 seasonal breeder, its annual reproductive cycle is divided into four stages: spermatogenesis,  
103 spawning, post-spawning, and resting or involution; this last stage occurs only when fish  
104 are ready to undergo sex change. During the resting or involution stages, the testicular area  
105 is characterized by a densely populated tissue, mainly formed by spermatogonia and Sertoli  
106 cells whose high rates of proliferation guaranty the next gametogenic stage (Chaves-Pozo et  
107 al., 2005a; Liarte et al., 2007). Leukocytes are located in the gonad and have a prominent  
108 role in the reproductive physiology (Chaves-Pozo et al., 2009; Chaves-Pozo et al., 2003,  
109 2005a; Liarte et al., 2007). Interestingly, the disruption of the reproductive functions due to  
110 natural and synthetic estrogen exposure alters the main activities of leukocytes, their  
111 recruitment into the gonad and the expression of immune relevant-molecules in the gonad  
112 (Cabas et al., 2011; Liarte et al., 2011a, b, c).

113           In this framework, we have firstly addressed whether Cim acts on HRH1 and HRH2  
114 in gilthead seabream leukocytes, and secondly we analysed the presence of histamine and  
115 the expression of HRH1, HRH2 and HRH3 coding genes in the gonad and the ability of  
116 Cim to regulate some reproductive functions, mainly cell renewal and steroidogenesis.

## 117 **2. Materials and Methods**

### 118 **2.1. Animals and experimental design**

119 Healthy gilthead seabream (*Sparus aurata* L., Actinopterygii, Perciformes, Sparidae) fish  
120 were bred and kept at the Centro Oceanográfico de Murcia (IEO, Mazarrón, Murcia). The

121 fish were kept in 0.17 m<sup>3</sup> tanks with natural water temperature with a flow-through circuit,  
122 suitable aeration and filtration system and natural photoperiod.

123 To test the effect of Cim (purity 98%; Sigma, St. Louis, USA) in the testicular  
124 physiology, 50 one year old fish (98.51±0.45 g body mass, BM) and 50 two years old fish  
125 (155.27±3.18 g BM) of gilthead seabream males at the resting stage were used (Fig. 1). The  
126 environmental parameters, mortality and food intake, were recorded daily. Cim was  
127 intraperitoneally (ip) injected at doses of 0 (control), 50, 100 or 200 mg/kg BM in PBS as a  
128 vehicle. The fish were kept and fed *ad libitum* three times at day and fasted during 24 h  
129 before sampling which was carried out at days 3 and 5 after Cim injection (days post-  
130 injection, dpi). Two hours prior sampling, all the fish (n=4 fish/Cim concentration and  
131 sampling time) were ip injected with 50 mg/kg BM of 5-bromo-2'-deoxyuridine (BrdU,  
132 Sigma, St. Louis, USA). Afterwards, fish were tranquilized with 20 µL/L of clove oil and,  
133 immediately, anesthetized using 40 µL/L of clove oil, weighed, decapitated, and blood,  
134 gonad and head kidney were removed. The serum samples were obtained from trunk blood  
135 by centrifugation (10,000 g, 1 min, 4°C) and immediately frozen and stored at -80°C. The  
136 gonads were weighed and processed for light microscopy and gene expression analysis, and  
137 head kidneys were used to obtain cell suspensions, as described below.

138 The experiments described comply with the Guidelines of the European Union  
139 Council (2010/63/EU), the Bioethical Committee of the University of Murcia (Spain) and  
140 the Instituto Español de Oceanografía (Spain) for the use of laboratory animals.

## 141 **2.2. Reactive oxygen intermediates production assay**

142 The head kidneys were washed in sRPMI medium (RPMI-1640 culture medium (Life  
143 Technologies, Madrid, Spain) adjusted to gilthead seabream serum osmolarity (353.33

144 mosmol) with 0.35% NaCl) and supplemented with 100 units/mL penicillin and 100 µg/mL  
145 streptomycin (P/S, Life Technologies, Madrid, Spain). Cell suspensions were obtained by  
146 forcing fragments of the organ through a nylon mesh (mesh size 100 µm). In order to  
147 determine whether Cim acts as an agonist or antagonist of Hrh1 or Hrh2, aliquots of 10<sup>6</sup>  
148 cells in 100 µL sRPMI containing 1% of fetal bovine serum (FBS, Life Technologies,  
149 Madrid, Spain) were incubated in the presence of 0.1 mM histamine (Sigma, St. Louis,  
150 USA), pyridilethylamine (Peth, a Hrh1 agonist, Sigma, St. Louis, USA), dimaprit (Dm, a  
151 Hrh2 agonist, Sigma, St. Louis, USA), 1 mM Cim or medium sRPMI alone during 1 hour  
152 previously to reactive oxygen intermediates (ROIs) production measurement. Similarly,  
153 aliquots of 10<sup>6</sup> cells in 100 µL sRPMI containing 1% of FBS (Life Technologies, Madrid,  
154 Spain) were incubated in the presence or absence of 0.1 mM Cim, and 10 minutes later 1  
155 mM chlorpheniramine (Chr, a Hrh1 antagonist, Sigma, St. Louise, USA) or famotidine  
156 (Fam, a Hrh2 antagonist, Sigma, St. Louise, USA) were added to the culture, which were  
157 incubated during 1 hour previously to ROIs production measurement. The ROIs production  
158 was then measured as the luminol-dependent chemiluminescence triggered by phorbol  
159 myristate acetate (PMA, Sigma, St. Louis, USA) (Mulero et al., 2001). Cell viability was  
160 checked in parallel samples by flow cytometry analysis of cells stained with 40 µg/mL  
161 propidium iodide in all treatments.

### 162 **2.3. Analysis of gene expression**

163 Mammals and fish HRH1 gene sequences (*Danio rerio* NM\_001042731.1; *Dicentrarchus*  
164 *labrax* CBN80867.1; *Bos taurus* NP\_776508; *Equus caballus* NP\_001075388; *Cricetulus*  
165 *griseus* ERE66926; *Mus musculus* AAK71654; *Pan troglodytes* NP\_001107637; *Rattus*  
166 *norvegicus* AAK71644; *Homo sapiens* NP\_000852; *Macaca fascicularis* EHH51138;



167 *Macaca mulatta* EHH16174) were used to determine a conserved region useful to design  
168 primers (Table 1) and clone a partial sequence of the *hrh1* of gilthead seabream using PCR  
169 techniques and head kidney cDNA as a template. The sequence obtained was launched  
170 using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) within teleost databases  
171 to verify its identity (Table 2). The sequence obtained was published at the GenBank  
172 database with an accession number LN875558 and protein sequence accession number  
173 CTQ87325.1. Then, specific primers for gene expression analysis were design (Table 1).  
174 The *hrh2* and *hrh3* were identified after blast searches in the IATS-CSIC transcriptomic  
175 database ([www.nutrigroup-iats.org/seabreamdb](http://www.nutrigroup-iats.org/seabreamdb)). Partial sequences of 932 and 1815  
176 nucleotides in length were unequivocally annotated as *hrh2* (E = 5e-50) and *hrh3* (E = 9e-  
177 169), respectively. The resulting nucleotide sequences were uploaded to GenBank with the  
178 accession numbers KP728255 (*hrh2*) and KP728256 (*hrh3*).

179 Total RNA was extracted from gonad fragments with TRIzol Reagent (Life  
180 Technologies, Madrid, Spain). Gonads were homogenised in 1mL of Trizol in an ice bath,  
181 and mixed with 200 µL of chloroform. The suspension was then centrifuged at 12,000 xg  
182 for 15 min. The clear upper phase was aspirated and placed in a clean tube. Five hundred  
183 microliters of isopropanol were then added, and the samples were again centrifuged at  
184 12,000 xg for 10 min. The RNA pellet was washed with 75% ethanol, dissolved in  
185 diethylpyrocarbonate (DEPC)-treated water and stored at -80 °C. The RNA was quantified  
186 with a spectrophotometer (Cecil Instruments Ltd) and the amount of RNA define as one  
187 unit of absorbance at 260 nm correspond to 40 µg/µL de RNA. The absorbance at 280 and  
188 320 was also analyzed to determine the grade of purity of total RNA. One µg of the total  
189 RNA was then treated with DNase I (amplification grade, 1 unit/µg RNA, Life

190 Technologies, Madrid, Spain) to remove genomic DNA traces that might interfere in the  
191 PCR reactions, and the SuperScript III RNase H–Reverse Transcriptase (Life  
192 Technologies, Madrid, Spain) was used to synthesize first strand cDNA with oligo-dT18  
193 primer at 50°C for 60 min.

194 Real-time PCR was used to analyse the expression of the genes coding for  
195 histamine receptor H1 (*hrh1*), H2 (*hrh2*) and H3 (*hrh3*), the testicular specific protein,  
196 double sex-and mab3-related transcription factor 1 (*dmrt1*), and some testicular  
197 steroidogenesis-related molecules as (i) the steroidogenic acute regulatory protein (*star*),  
198 (ii) the cholesterol side chain cleavage cytochrome P450 (*cyp11a1*), (iii) the steroid 11-  
199 beta-hydroxylase (*cyp11b1*), and (iv) the aromatase (*cyp19a1a*). Real-time PCR was  
200 performed with an ABI PRISM 7500 instrument (Life Technologies, Madrid, Spain) using  
201 SYBR Green PCR Core Reagents (Life Technologies, Madrid, Spain) as previously  
202 described (Chaves-Pozo et al., 2008b). For each sample, gene expression levels were  
203 corrected by the *ribosomal protein S18* gene (*rps18*) content presented as  $2^{-\Delta Ct}$ , where  $\Delta Ct$   
204 is determined by subtracting the *rps18* Ct value from the target Ct. The gilthead seabream  
205 specific primers used are shown in Table 1. In all cases, each PCR was repeated at least  
206 twice. Less than 2% variation in *rps18* gene expression was observed between samples.  
207 Before the experiments, the specificity of each primer pair was studied using positive and  
208 negative samples. A melting curve analysis of the amplified products validated the primer  
209 for specificity. Negative controls with no template were always included in the reactions.

#### 210 **2.4. Light microscopy and immunocytochemical staining**

211 Gonads, fixed in Bouin's fixative solution or 4% paraformaldehyde in PBS (PAF), were  
212 embedded in paraffin (Paraplast Plus; Sherwood Medical, Athy, Ireland), and sectioned at 5

213  $\mu\text{m}$ . After dewaxing and rehydratation, some sections were stained with haematoxylin-  
214 eosin in order to determine the developmental state of each animal. Some serial sections  
215 fixed with Bouin's solution were subjected to an indirect immunocytochemical method  
216 using two antibodies: a polyclonal antibody (pAb) against histamine (Sigma) which has  
217 been previously used in the characterization of mast cells of gilthead seabream (Mulero et  
218 al., 2007) and a monoclonal Ab (mAb) specific to gilthead seabream acidophilic  
219 granulocytes (G7) (Sepulcre et al., 2002) which has been previously used in the  
220 characterization of testicular AGs (Chaves-Pozo et al., 2005b). Some sections fixed with  
221 PAF were subjected to an indirect immunocytochemical method using a mAb specific to  
222 BrdU (Becton Dickinson, San Jose, USA), at the optimal dilution of 1:100, that revealed  
223 the proliferative cells which have incorporated the BrdU, previously injected, during their  
224 DNA synthesis phase. Negative controls were done by omitting the first antiserum or in the  
225 case of the BrdU detection by using tissue sections from fish that had not been injected with  
226 BrdU. Some other sections fixed with PAF were subjected to *in situ* detection of DNA  
227 fragmentation (TUNEL) assay to identify apoptotic cells (*in situ* cell death detection kit;  
228 Roche, Basel, Switzerland) (Chaves-Pozo et al., 2007). Negative controls were processed in  
229 an identical manner, except that the TdT enzyme was omitted. Positive controls were also  
230 performed treating the sections with DNase I (3–3000 U/mL; Sigma) in 50 mM Tris-HCl  
231 (pH 7.5), 10 mM  $\text{MgCl}_2$ , and BSA (1 mg/mL) for 10 min at room temperature to induce  
232 DNA strand breaks before labelling. Positive controls for anti-histamine immunostaining  
233 were performed using gut sections, in which histamine positive cells has been described in  
234 the connective tissue (Mulero et al., 2007). Positive controls for G7 immunostaining was

235 performed using head-kidney sections, in which AGs cells has been described in the  
236 myelopoiesis areas of the tissue (Chaves-Pozo et al., 2005c).

237 Slides were examined with an Eclipse E600 (Nikon) light microscope using 200, 400 and  
238 600x magnifications. The images were obtained with an Olympus SC30 digital camera  
239 (Olympus soft imaging solutions GMBH) and Spot 3.3 software (Diagnostic instruments,  
240 Inc).

## 241 **2.5. Analytical techniques**

242 Serum levels of 17 $\beta$ -estradiol (E<sub>2</sub>) and 11-ketotestosterone (11KT) were quantified by  
243 ELISA, following the method previously used in gilthead seabream (Chaves-Pozo et al.,  
244 2008a). Steroids were extracted from 30  $\mu$ L of serum in 1.3 mL of methanol (Panreac,  
245 Barcelona, Spain). Then, methanol was evaporated at 37°C and the steroids were  
246 resuspended in 600  $\mu$ L of reaction buffer [0.1 M phosphate buffer with 1 mM EDTA  
247 (Sigma, St. Louis, USA), 0.4 M NaCl (Sigma, St. Louis, USA), 1.5 mM NaN<sub>3</sub> (Sigma, St.  
248 Louis, USA) and 0.1% albumin from bovine serum (Sigma, St. Louis, USA)]. Of this  
249 solution, 50  $\mu$ L were used for each ELISA reaction (2.5  $\mu$ L of serum per reaction). A  
250 standard curve from 6.13 x 10<sup>-4</sup> to 5 ng/mL (0.03-250 pg/well), a blank and a non specific  
251 binding control (negative control) were established in all the assays. E<sub>2</sub> and 11KT  
252 standards, mouse anti-rabbit IgG mAb, and specific anti-steroid antibodies and enzymatic  
253 tracers (steroid acetylcholinesterase conjugates) were obtained from Cayman Chemical.  
254 Microtiter plates (MaxiSorp) were purchased from Nunc. The reaction was revealed using  
255 Ellman's reagent (Cayman Chemical) and the absorbance of the samples was measured at  
256 405 nm every 2 min intervals during 5 h at 25 °C using a Thermo Scientific Multiskan GO  
257 plate reader. Standards and extracted plasma samples were run in duplicate and all the

258 readings were corrected with the blank and negative control. The lower detection limit for  
259 all the assays was 12.2 pg/mL. The inter-assay coefficients of variation at 50% of binding  
260 were 6.4% for E<sub>2</sub> (n=3) and 4.8% for 11KT (n=3). The intra-assay coefficients of variation  
261 (calculated from sample duplicates) were  $8.2 \pm 2.3\%$  for E<sub>2</sub> and  $6.9 \pm 1.3\%$  for 11KT  
262 assays. Details on cross-reactivity for specific antibodies were provided by the supplier  
263 (0.01% of anti-11KT reacts with T; and 0.1% of anti-E<sub>2</sub> reacts with T).

## 264 **2.6. Calculation and statistics**

265 The gonadosomatic index (GSI) was calculated as an index of the reproductive stage [ $100 \times$   
266 (GM/BM) (%)] where GM is gonad mass (in grams) and BM is body mass (in grams).

267 The quantification of anti-BrdU immunostained and TUNEL stained indexes were  
268 calculated as the mean value  $\pm$  SEM of the stained area/total area of 24 randomly  
269 distributed optical areas at 200 x magnification. The stained areas were measured by image  
270 analysis using a Nikon eclipse E600 light microscope, an Olympus SC30 digital camera  
271 (Olympus soft imaging solutions GMBH), and Image Tool 3.00 software (The University  
272 of Texas Health Science Center).

273 Data were analysed by one-way ANOVA and a post hoc test (Tukey Honestly  
274 Significant Difference or Waller Duncan) to determine differences between groups  
275 ( $P \leq 0.05$ ). Normality of the data was previously assessed using a Shapiro–Wilk test and  
276 homogeneity of variance was also verified using the Levene test. All data related to sex  
277 steroid serum levels and gene expressions did not meet parametric assumptions, and they  
278 were subjected to a non-parametric Kruskal–Wallis test, followed by a multiple comparison  
279 test. The critical value for statistical significance was taken as  $P \leq 0.05$ . Statistical analyses

280 were conducted using SPSS 12.0 (SPSS, Chicago, IL, USA). All data are presented as  
281 mean  $\pm$  standard error to the mean (SEM).

### 282 **3. Results**

#### 283 *3.1. Identification of a partial sequence of hrh1 gene*

284 A partial sequence of the *hrh1* gene of 295 base pair have been cloned and published in the  
285 genebank with the accession number LN875558. This sequence coded for a 98 amino acid  
286 peptide that had a 93% of homology with the *hrh1* gene of several teleosts species (Table  
287 2). Moreover, this sequence coded for 7 transmembrane receptor (rhodopsin family)  
288 domain, which is present in the most of the HRH1 orthologes.

#### 289 *3.2. Cim acts as an agonist of HRH1 and HRH2 in gilthead seabream leukocytes*

290 First of all, the ability of Cim to act through gilthead seabream HRH1 or HRH2 by  
291 determining the modification on the ROIs production of head kidney leukocytes in the  
292 presence or absence of specific HR agonists or antagonists was analysed. The results  
293 showed that 1 mM Cim increased leukocyte ROI production as 0.1 mM His did, while 0.1  
294 mM of Peth (HRH1 agonist) or 0.1 mM of Dm (HRH2 agonist) failed to do so (Fig. 2a).  
295 However, 1 mM Chr (HRH1 antagonist) or Fam (HRH2 antagonist) inhibited or stimulated  
296 the ROI production, respectively (Fig. 2b). When the cells were incubated with 0.1 mM  
297 Cim in the presence of 1 mM Chr or Fam, both antagonist inhibited the Cim-stimulated  
298 ROIs production (Fig. 2b). The assessment of cell viability, as assayed by propidium iodide  
299 staining, demonstrated that this parameter was not significantly affected by the treatments  
300 employed. The non staining cells range between  $95.89 \pm 0.22$  and  $96.54 \pm 0.23\%$  of total

301 cells for all compounds except for 1 mM Chr, which reduced cell viability to  $94.88 \pm$   
302 0.37%.

303 *3.3. The hrh1, hrh2 and hrh3 genes are expressed in the gonad although their expressions*  
304 *were differently modified by Cim*

305 All three HRs were expressed in the gonad of gilthead seabream (Fig. 3). In 1 year old fish,  
306 their expression levels were not modified by Cim treatment (Fig. 3a,c,e). However, in 2  
307 year old fish *hrh1* was up-regulated at 5 dpi of 50 mg Cim/kg BM treatment (Fig. 3b),  
308 while the expression of *hrh2* and *hrh3* were up-regulated at 5 dpi of 200 mg Cim/kg BM  
309 treatment (Fig. 3d,f).

310 *3.4. Histamine positive cells were present in the testis*

311 Histamine was immunodetected in some interstitial cells of the testis (Fig 4a) and in the  
312 connective tissue that limited the testicular and the ovarian areas and around the efferent  
313 ducts (Fig. 4b). These cells are granular cells that differ from acidophilic granulocytes (Fig.  
314 4c), as previously described (Mulero et al., 2007), and as has been confirmed by negative  
315 (Fig. 4d, for histamine) and positive (Fig. 4 e, for histamine; f, for G7) controls.

316 *3.5. Cim differentially affects the GSI, the testicular cell proliferation and apoptotic rates*  
317 *and the dmrt1 gene expression in both ages analysed*

318 Cim treatment was unable to affect the GSI in 1 year old fish while diminished it in 2 years  
319 old fish (5 dpi with all Cim concentrations assayed) (Table 3).

320 BrdU and TUNEL staining determined that the Cim altered the renewal rates of  
321 testicular cells. Thus, the BrdU immunostaining index decreased (at 3 dpi with 100 and 200  
322 mg Cim/kg BM) or increased (at 5 dpi with all the Cim concentrations assayed) in the

323 gonad of 1 year old males (Table 3). However, this index decreased in the gonad of 2 years  
324 old males (at 5 dpi with all the concentrations assayed) (Table 3). Interestingly, the size,  
325 morphology and localization of the nuclei immunostained with anti-BrdU determined that  
326 although in control fish both germ and Sertoli cells proliferate, Cim increase the Sertoli cell  
327 proliferation (Fig. 5a,b,c). In the other hand, the TUNEL staining index diminished in the  
328 testis of 1 year old males (at 3 dpi with 200 mg Cim/kg BM and at 5 dpi with 50 mg  
329 Cim/kg BM) and in 2 years old males (at all times and Cim concentrations assayed) except  
330 after 5 dpi of 100 mg Cim/kg BM treatment where an increase was recorded (Table 3).  
331 Most of the apoptotic cells resembled the location and the nuclear shape characteristic of  
332 Sertoli cells in both control and Cim treated testis, although some spermatogonia were also  
333 observed (Fig. 5d,e).

334 *Dmrt1* gene expression was down-regulated in the gonad of 1 and 2 years old males  
335 with 100 mg Cim/kg BM, although the exposure time needed varied with the age of the fish  
336 (5 dpi in 1 year old animals, Fig. 6a, and 3 dpi in 2 years old animals, Fig. 6b, respectively).

### 337 *3.6. Cim differentially modifies testicular steroidogenesis depending on the fish age*

338 Cim altered differentially the E<sub>2</sub> and 11KT serum levels of 1 and 2 years old gilthead  
339 seabream males. Regarding the E<sub>2</sub> serum levels, an increase was observed in fish of both  
340 ages, although the Cim concentration and the exposure time needed varied between both  
341 groups (at 3 dpi with 100 and 200 mg Cim/kg BM in 1 year old animals, Fig. 6c, and with  
342 all Cim concentration assayed in 2 years old animals and at 5 dpi with 100 and 200 mg  
343 Cim/Kg BM in 2 years old animals, Fig. 6d). In contrast, 11KT serum levels decreased in 1  
344 year old males (at 3 dpi with 200 mg Cim/kg BM and at 5 dpi with 100 and 200 mg Cim/kg



345 BM, Fig. 6e), while increased in 2 years old males (at 3 dpi with all the Cim concentrations  
346 used, Fig. 6f).

347 Differences in the transcriptional levels of several steroidogenic molecule genes in  
348 the gonad were recorded between 1 and 2 years old males after Cim injection (Fig. 7). In 1  
349 year old fish, *star*, *cyp11a1* and *cyp11b1* gene expression were down-regulated (*star* at 3  
350 dpi with all the concentrations assayed and at 5 dpi with 100 and 200 mg Cim/kg BM, Fig.  
351 7a; *cyp11a1* at 3 dpi with 50, 100 and 200 mg Cim/kg BM and at 5 dpi with 50 and 100 mg  
352 Cim/kg BM, Fig. 7c; and *cyp11b1* at 3 dpi with 50 and 200 mg Cim/kg BM and at 5 dpi  
353 with 100 and 200 mg Cim/kg BM, Fig. 7e). However, no differences were observed in the  
354 expression pattern of *cyp19a1a* gene (Fig. 7g). In the other hand, in the gonad of 2 years  
355 old males, the transcription of *star* and *cyp11b1* was up-regulated (5 dpi with 50 mg  
356 Cim/kg BM, Fig. 7b,f, respectively) and the transcription of *cyp11a1* and *cyp19a1a* was  
357 down regulated (*cyp11a1* at 3 and 5 dpi with 100 mg Cim/kg BM, Fig. 7d, and *cyp19a1a* at  
358 3 dpi with 50 mg Cim/kg BM and at 5 dpi with 200 mg Cim/kg BM, Fig. 7h).

#### 359 **4. Discussion**

360 Our data demonstrate that Cim is able to differentially modify the cell renewal rates and the  
361 steroidogenesis of gilthead seabream, a hermaphrodite fish species of important  
362 commercial value in the Mediterranean area. Moreover, the alteration observed depended  
363 on the maturational stage of the fish. These data allow us to consider the fish population as  
364 a potential target for this pharmacological compound that has been detected in the surface  
365 watercourses and recently in a coastal marine lagoon (Mar Menor, Murcia, Spain) (Kolpin  
366 et al., 2002; Moreno-González et al., 2014; Ternes et al., 2001). Although nothing is known  
367 about Cim concentration in open marine sea waters, Cim is a pharmaceutical compound

368 consumes all over the world. The assessment of how Cim can affect marine fish species  
369 became important in order to determine the needed of future constrain measures mainly in  
370 areas with low exchange water flow or with intensive aquaculture activity.

371         In mammals, Cim has been reported to be an antagonist of HRH2 with virtually no  
372 affinity for HRH1 and a very weak affinity for HRH3 (van der Goot and Timmerman,  
373 2000). We have previously demonstrated that histamine is biologically active in gilthead  
374 seabream leukocytes where it is able to regulate the inflammatory response by acting on  
375 professional phagocytes mediating through the engagement of HRH1 and HRH2 (Mulero et  
376 al., 2007). Our data demonstrated now that Cim is able to act through gilthead seabream  
377 HRH1 and HRH2 modulating the ROI production of head kidney leukocytes. Thus, Cim,  
378 when administrated alone, has similar effects than histamine. Moreover, when leukocytes  
379 were incubated with Cim in the presence of specific HRH1 or HRH2 antagonist (Chr or  
380 Fam, respectively), Cim-induced ROIs production was inhibited or down regulated. Our  
381 data determined that Cim acts through HRH1 receptor, as Chr decreased Cim stimulated  
382 ROIs production to control levels, but also through HRH2 receptors as Fam down regulated  
383 the Cim stimulated ROIs production to the same levels of Fam treated group. That means  
384 that part of the action of Cim is being blockaged by Fam. All these data disclose that Cim  
385 exerts its effects on gilthead seabream leukocytes by means of HRH1 and HRH2  
386 interaction. In addition, further studies to clearly characterize the HRH1 and HRH2 binding  
387 affinities in gilthead seabream will help in understanding their individual contribution in the  
388 Cim/histamine mediated response.

389         In mammals and reptiles, histamine has been shown to modulate testicular  
390 steroidogenesis through HRH1 and HRH2 in a biphasic manner depending upon its

391 concentration (Albrecht et al., 2005; Khan and Rai, 2007; Mondillo et al., 2005). In fact,  
392 testicular mast cells has been described as a potential source of histamine and HRH1 and  
393 HRH2 have been detected in all compartment of the human testis (Albrecht et al., 2004).  
394 Moreover, the alterations observed on histamine descarboxilase-deficient knockout mice  
395 indicate that peripheral histamine is an important factor in male gonad development (Pap et  
396 al., 2002). We have demonstrated for the first time that histamine is stored in a cell type  
397 located in the connective tissue between the seminiferous tubules, different to the  
398 acidophilic granulocytes, as it has been previously described in the gills and intestine of  
399 gilthead seabream (Mulero et al., 2007). Moreover, HRs (*hrh1*, *hrh2* and *hrh3*) are  
400 expressed in the gonad of this hermaphrodite species although at very low levels.  
401 Moreover, our data suggest that Cim acts directly on the gonad of gilthead seabream  
402 although further studies will be needed in order to exactly determine its effect on cell  
403 renewal and steroidogenesis. Our *in vivo* data clearly point to Cim as an endocrine disruptor  
404 for gilthead seabream and probably for other fish species, although further studies will be  
405 needed to determine the regulatory mechanisms involved in the testicular disruption  
406 produced by Cim. Thus Cim affected the testicular physiology by promoting an increase on  
407 cell proliferation in the gonad of 1 year old fish and a decrease in the gonad of 2 years old  
408 fish. Interestingly, Cim down-regulated the apoptotic rates in gilthead seabream while in  
409 mammals Cim induces the apoptosis of peritubular cells producing, in turn, the detachment  
410 and apoptosis of Sertoli and germ cells (Sasso-Cerri and Cerri, 2008; Sasso-Cerri and  
411 Miraglia, 2002). The main cell type affected by Cim in the gilthead seabream testis were  
412 Sertoli cells, although the affection of other cell types such as peritubular or Leydig cells  
413 cannot be discarded. It is worth to note that Sertoli cells lost their capability to proliferate in  
414 the mature mammalian testis while in teleosts, Sertoli cells proliferate in mature fish testis

415 (Chaves-Pozo et al., 2005a; Hess, 1999; Schulz et al., 2012). This could explain why the  
416 main effect observed in the mammalian testis upon Cim treatment so far is related to  
417 apoptosis of several testicular cell types (Franca et al., 2000; Sasso-Cerri and Cerri, 2008;  
418 Sasso-Cerri and Miraglia, 2002). Moreover, and in contrast to mammals, in the gilthead  
419 seabream, the changes observed in the renewal (proliferation and apoptosis) of the  
420 testicular cells upon Cim treatment, did not compromise the functionality of the testis as  
421 indicated by the slight recorded alteration in the *dmrt1* gene expression, a gene related to  
422 testicular maintenance in fish (Marchand et al., 2000). Although further studies will be  
423 needed, our data suggest that the effect of Cim on reproduction in fish are not so toxic as  
424 reported in mammals, probably due to the ability of the different cell types of the fish testis  
425 to proliferate after puberty.

426 In mammals, histamine induces a dual concentration-dependent effect on Leydig  
427 cell steroidogenesis through HRH1 and HRH2 activation (Mondillo et al., 2009). In the  
428 gilthead seabream, Cim treatment triggered an increase in E<sub>2</sub> serum levels at both ages  
429 analysed while the 11KT levels were down-regulated in 1 year old fish and up-regulated in  
430 2 years old fish. The effect on 11KT serum levels observed in 1 year old fish correlated  
431 with the down-regulation of gene expressions involved in androgen production, while  
432 *cyp19a1a* gene expression was unaltered. Moreover, despite of the changes observed in E<sub>2</sub>  
433 and 11KT serum levels in 2 years old fish, the expression the genes coding for  
434 steroidogenetic molecules scarcely varied in the gonad. Interestingly, differences between 1  
435 and 2 years old fish were observed in all the processes studied, suggesting that the role of  
436 Cim in reproductive tissues depends on fish maturity, as one year old fish at resting stage

437 are still pre-puberal fish, while two years old fish at resting stage have gone through one  
438 spawning season (Chaves-Pozo et al., 2009; Chaves-Pozo et al., 2005a).

## 439 **5. Conclusion**

440 In conclusion, our data clearly demonstrate that Cim acts through Hrh1 and Hrh2 in  
441 gilthead seabream head kidney leukocytes and that these receptors coding genes, together  
442 with the Hrh3, are expressed in the gonad of this species, as well as histamine is present in  
443 the gonad. Cim might act by Hrs to trigger the effects observed on testicular cell renewal  
444 and steroidogenesis. Interestingly, and although some effects of Cim observed in fish are  
445 less pronounced than those described in mammals, Cim showed a clear disrupter effect on 1  
446 and 2 years old gilthead seabream fish.

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## 455 **9. References**

456 Albrecht, M., Frungieri, M.B., González-Calvar, S., Meineke, V., Kohn, F.M., Mayerhofer,  
457 A., 2005. Evidence for a histaminergic system in the human testis. *Fertil Steril* 83, 1060-  
458 1063.  
459 Cabas, I., Chaves-Pozo, E., García-Alcázar, A., Meseguer, J., Mulero, V., García-Ayala,  
460 A., 2011. Dietary intake of 17alpha-ethinylestradiol promotes leukocytes infiltration in the  
461 gonad of the hermaphrodite gilthead seabream. *Mol Immunol* 48, 2079-2086.

- 462 Chaves-Pozo, E., Arjona, F.J., García-López, A., García-Alcázar, A., Meseguer, J., García-  
463 Ayala, A., 2008a. Sex steroids and metabolic parameter levels in a seasonal breeding fish  
464 (*Sparus aurata* L.). *Gen Comp Endocrinol* 156, 531-536.
- 465 Chaves-Pozo, E., Liarte, S., Fernández-Alacid, L., Abellán, E., Meseguer, J., Mulero, V.,  
466 García-Ayala, A., 2008b. Pattern of expression of immune-relevant genes in the gonad of a  
467 teleost, the gilthead seabream (*Sparus aurata* L.). *Mol Immunol* 45, 2998-3011.
- 468 Chaves-Pozo, E., Liarte, S., Mulero, I., Abellán, E., Meseguer, J., García-Ayala, A., 2009.  
469 Early presence of immune cells in the developing gonad of the gilthead seabream (*Sparus*  
470 *aurata* Linnaeus, 1758). *J Reprod Dev* 55, 440-445.
- 471 Chaves-Pozo, E., Liarte, S., Vargas-Chacoff, L., García-López, A., Mulero, V., Meseguer,  
472 J., Mancera, J.M., García-Ayala, A., 2007. 17Beta-estradiol triggers postspawning in  
473 spermatogenically active gilthead seabream (*Sparus aurata* L.) males. *Biol Reprod* 76, 142-  
474 148.
- 475 Chaves-Pozo, E., Mulero, V., Meseguer, J., García-Ayala, A., 2005a. An overview of cell  
476 renewal in the testis throughout the reproductive cycle of a seasonal breeding teleost, the  
477 gilthead seabream (*Sparus aurata* L.). *Biol Reprod* 72, 593-601.
- 478 Chaves-Pozo, E., Mulero, V., Meseguer, J., García-Ayala, A., 2005b. Professional  
479 phagocytic granulocytes of the bony fish gilthead seabream display functional adaptation to  
480 testicular microenvironment. *J Leukoc Biol* 78, 345-351.
- 481 Chaves-Pozo, E., Muñoz, P., López-Muñoz, A., Pelegrín, P., García-Ayala, A., Mulero, V.,  
482 Meseguer, J., 2005c. Early innate immune response and redistribution of inflammatory  
483 cells in the bony fish gilthead seabream experimentally infected with *Vibrio anguillarum*.  
484 *Cell Tissue Res* 320, 61-68.
- 485 Chaves-Pozo, E., Pelegrín, P., Mulero, V., Meseguer, J., García-Ayala, A., 2003. A role for  
486 acidophilic granulocytes in the testis of the gilthead seabream (*Sparus aurata* L., Teleostei).  
487 *J Endocrinol* 179, 165-174.
- 488 França, L.R., Leal, M.C., Sasso-Cerri, E., Vasconcelos, A., Debeljuk, L., Russell, L.D.,  
489 2000. Cimetidine (Tagamet) is a reproductive toxicant in male rats affecting peritubular  
490 cells. *Biol Reprod* 63, 1403-1412.
- 491 Hess, R.A., 1999. Spermatogenesis, overview, in: Knobil, E., Neil, J.D. (Eds.),  
492 *Encyclopedia of reproduction*. Academic press, New York, pp. 539-545.
- 493 Hosseinifard, S. M., Ahmadvpour, A., Mojazi Amiri, B., Razeghi Mansour, M.,  
494 Ebrahimpour, A. 2013. Immunomodulatory effect of cimetidine in common carp (*Cyprinus*  
495 *carpio* L.). *Fish Physiol Biochem* 39, 1505-1511.
- 496 Khan, U.W., Rai, U., 2007. Differential effects of histamine on Leydig cell and testicular  
497 macrophage activities in wall lizards: precise role of H1/H2 receptor subtypes. *J Endocrinol*  
498 194, 441-448.
- 499 Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B.,  
500 Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater  
501 contaminants in U.S. streams, 1999-2000: a national reconnaissance. *Environ Sci Technol*  
502 36, 1202-1211.
- 503 Kubecova, M., Kolostova, K., Pinterova, D., Kacprzak, G., Bobek, V., 2011. Cimetidine:  
504 an anticancer drug? *Eur J Pharm Sci* 42, 439-444.
- 505 Kubota, T., Fujiwara, H., Ueda, Y., Itoh, T., Yamashita, T., Yoshimura, T., Okugawa, K.,  
506 Yamamoto, Y., Yano, Y., Yamagishi, H., 2002. Cimetidine modulates the antigen  
507 presenting capacity of dendritic cells from colorectal cancer patients. *Br J Cancer* 86, 1257-  
508 1261.

- 509 Lee, S., Jung, D., Kho, Y., Ji, K., Kim, P., Ahn, B., Choi, K., 2015. Ecotoxicological  
510 assessment of cimetidine and determination of its potential for endocrine disruption using  
511 three test organisms: *Daphnia magna*, *Moina macrocopa*, and *Danio rerio*. *Chemosphere*  
512 135, 208–216.
- 513 Liarte, S., Cabas, I., Chaves-Pozo, E., Arizcun, M., Meseguer, J., Mulero, V., García-  
514 Ayala, A., 2011a. Natural and synthetic estrogens modulate the inflammatory response in  
515 the gilthead seabream (*Sparus aurata* L.) through the activation of endothelial cells. *Mol*  
516 *Immunol* 48, 1917-1925.
- 517 Liarte, S., Chaves-Pozo, E., Abellán, E., Meseguer, J., Mulero, V., Canario, A.V., García-  
518 Ayala, A., 2011b. Estrogen-responsive genes in macrophages of the bony fish gilthead  
519 seabream: A transcriptomic approach. *Dev Comp Immunol* 35, 840-849.
- 520 Liarte, S., Chaves-Pozo, E., Abellán, E., Meseguer, J., Mulero, V., García-Ayala, A.,  
521 2011c. 17beta-Estradiol regulates gilthead seabream professional phagocyte responses  
522 through macrophage activation. *Dev Comp Immunol* 35, 19-27.
- 523 Liarte, S., Chaves-Pozo, E., García-Alcázar, A., Mulero, V., Meseguer, J., García-Ayala,  
524 A., 2007. Testicular involution prior to sex change in gilthead seabream is characterized by  
525 a decrease in DMRT1 gene expression and by massive leukocyte infiltration. *Reprod Biol*  
526 *Endocrinol* 5, 20-35.
- 527 Lo Nostro, F.L., Antoneli, F.N., Quagio-Grassiotto, I., Guerrero, G.A., 2004. Testicular  
528 interstitial cells, and steroidogenic detection in the protogynous fish, *Synbranchus*  
529 *marmoratus* (Teleostei, Synbranchidae). *Tissue Cell* 36, 221-231.
- 530 Marchand, O., Govoroun, M., D'Cotta, H., McMeel, O., Lareyre, J.J., Bernot, A., Laudet,  
531 V., Guiguen, Y., 2000. DMRT1 expression during gonadal differentiation and  
532 spermatogenesis in the rainbow trout, *Oncorhynchus mykiss*. *Biochim Biophys Acta* 1493,  
533 180-187.
- 534 Mayerhofer, A., Bartke, A., Amador, A.G., Began, T., 1989. Histamine affects testicular  
535 steroid production in the golden hamster. *Endocrinology* 125, 2212-2214.
- 536 Minucci, S., Vitiello, II, Marmorino, C., Di Matteo, L., Baccari, G.C., 1995. Mast cell-  
537 Leydig cell relationships in the testis of the lizard *Podarcis s. sicula* Raf: thermal  
538 manipulation, ethane 1,2-dimethane sulphonate (EDS) and sex hormone treatment. *Zygote*  
539 3, 259-264.
- 540 Mondillo, C., Pagotto, R.M., Piotrkowski, B., Reche, C.G., Patrignani, Z.J., Cymeryng,  
541 C.B., Pignataro, O.P., 2009. Involvement of nitric oxide synthase in the mechanism of  
542 histamine-induced inhibition of Leydig cell steroidogenesis via histamine receptor subtypes  
543 in Sprague-Dawley rats. *Biol Reprod* 80, 144-152.
- 544 Mondillo, C., Patrignani, Z., Reche, C., Rivera, E., Pignataro, O., 2005. Dual role of  
545 histamine in modulation of Leydig cell steroidogenesis via HRH1 and HRH2 receptor  
546 subtypes. *Biol Reprod* 73, 899-907.
- 547 Moreno-González, R., Rodríguez-Mozaz, S., Gros, M., Pérez-Cánovas, E., Barceló, D.,  
548 León, V.M., 2014. Input of pharmaceuticals through coastal surface watercourses into a  
549 Mediterranean lagoon (Mar Menor, SE Spain): sources and seasonal variations. *Sci Total*  
550 *Environ* 490, 59-72.
- 551 Mulero, I., Sepulcre, M.P., Meseguer, J., García-Ayala, A., Mulero, V., 2007. Histamine is  
552 stored in mast cells of most evolutionarily advanced fish and regulates the fish  
553 inflammatory response. *Proc Natl Acad Sci U S A* 104, 19434-19439.

- 554 Mulero, V., Pelegrín, P., Sepulcre, M.P., Muñoz, J., Meseguer, J., 2001. A fish cell surface  
555 receptor defined by a mAb mediates leukocyte aggregation and deactivation. *Dev Comp*  
556 *Immunol* 25, 619-627.
- 557 Pap, E., Racz, K., Kovacs, J.K., Varga, I., Buzas, E., Madarasz, B., Foldes, C., Szalai, C.,  
558 Watanabe, T., Ohtsu, H., Ichikawa, A., Nagy, A., Falus, A., 2002. Histidine decarboxylase  
559 deficiency in gene knockout mice elevates male sex steroid production. *J Endocrinol* 175,  
560 193-199.
- 561 Peitsaro, N., Sundvik, M., Anichtchik, O.V., Kaslin, J., Panula, P., 2007. Identification of  
562 zebrafish histamine H1, H2 and H3 receptors and effects of histaminergic ligands on  
563 behavior. *Biochem Pharmacol* 73, 1205-1214.
- 564 Sasso-Cerri, E., Cerri, P.S., 2008. Morphological evidences indicate that the interference of  
565 cimetidine on the peritubular components is responsible for detachment and apoptosis of  
566 Sertoli cells. *Reprod Biol Endocrinol* 6, 18.
- 567 Sasso-Cerri, E., Miraglia, S.M., 2002. In situ demonstration of both TUNEL-labeled germ  
568 cell and Sertoli cell in the cimetidine-treated rats. *Histol Histopathol* 17, 411-417.
- 569 Schulz, R.W., van Dijk, W., Chaves-Pozo, E., García-Lopez, A., de Franca, L.R., Bogerd,  
570 J., 2012. Sertoli cell proliferation in the adult testis is induced by unilateral gonadectomy in  
571 African catfish. *Gen Comp Endocrinol* 177, 160-167.
- 572 Sepulcre, M.P., Pelegrín, P., Mulero, V., Meseguer, J., 2002. Characterisation of gilthead  
573 seabream acidophilic granulocytes by a monoclonal antibody unequivocally points to their  
574 involvement in fish phagocytic response. *Cell Tissue Res* 308, 97-102.
- 575 Smit, M.J., Leurs, R., Alewijnse, A.E., Blauw, J., Van Nieuw Amerongen, G.P., Van De  
576 Vrede, Y., Roovers, E., Timmerman, H., 1996. Inverse agonism of histamine H2 antagonist  
577 accounts for upregulation of spontaneously active histamine H2 receptors. *Proc Natl Acad*  
578 *Sci U S A* 93, 6802-6807.
- 579 Takahashi, H.K., Watanabe, T., Yokoyama, A., Iwagaki, H., Yoshino, T., Tanaka, N.,  
580 Nishibori, M., 2006. Cimetidine induces interleukin-18 production through H2-agonist  
581 activity in monocytes. *Mol Pharmacol* 70, 450-453.
- 582 Ternes, T., Bonerz, M., Schmidt, T., 2001. Determination of neutral pharmaceuticals in  
583 wastewater and rivers by liquid chromatography-electrospray tandem mass spectrometry. *J*  
584 *Chromatogr A* 938, 175-185.
- 585 van der Goot, H., Timmerman, H., 2000. Selective ligands as tools to study histamine  
586 receptors. *Eur J Med Chem* 35, 5-20.
- 587
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## 589 9. Figure legends

590 **Figure 1:** Schematic illustration of the timing of the different reproductive stages of the  
591 gilthead seabream related to the age (years). Arrows indicate the timing of cimetidine  
592 injection. SG, spermatogenesis; S, spawning; PS, post-spawning; R, resting; TI, testicular  
593 involution. Data obtained from this manuscript and from Chaves-Pozo et al. (2005a, 2009)  
594 and Liarte et al. (2007).

595 **Figure 2:** Head kidney leukocyte suspensions were incubated during 1 h with: (a) 0.1 mM  
596 histamine (His), pyridylethylamine (Peth, a Hrh1 agonist), dimaprit (Dm, a Hrh2 agonist) or  
597 1 mM cimetidine (Cim) or medium sRPMI alone, or (b) 0.1 M Cim in the presence or  
598 absence of 1 mM chlorpheniramine (Chr, a Hrh1 antagonist) or Famotidine (Fam, a Hrh2  
599 antagonist) or medium sRPMI alone. The respiratory burst activity was then measured as  
600 the luminol-dependent chemiluminescence triggered by PMA (1  $\mu$ g/mL). Data are  
601 presented as mean  $\pm$  S.E.M. of quadruplicate cultures and are representative of four  
602 independent experiments. Letters determine significant differences between groups (\*P  $\leq$   
603 0.05).

604 **Figure 3:** Expression levels of genes that code for histamine-receptors (Hr) in the gonad of  
605 1 (a,c,e) and 2 (b,d,f) years old gilthead seabream fish at resting stage. The expression of  
606 *hrh1* (a,b), *hrh2* (c,d) and *hrh3* (e,f) in fish injected with 0 (control), 50, 100 or 200 mg  
607 Cim/kg body mass (BM) after 3 and 5 days of injection was analysed by real time PCR.  
608 Data represent the means  $\pm$  S.E.M. of duplicate samples corresponding to four independent  
609 fish. Asterisks indicate significant differences between treated and control groups (\*P  $\leq$   
610 0.05).

611 **Figure 4:** Gonad (a-d), gut (e) and head-kidney (f) sections of gilthead seabream of one  
612 year old at resting stage immunostained with anti-histamine antibody (a,b,e) or G7 serum

613 (c,f) were located in interstitial cells between seminiferous tubules (a) and in the connective  
614 (b,c) tissue of the gonad. Negative control (d) and gut section immunostained with anti-  
615 histamine serum (e) and head-kidney section immunostained with G7 antibody (f)  
616 determine the specificity of the antibodies used. Cells containing histamine (black arrows)  
617 are not immunostained with G7 serum (white arrows and surrounded with a dark line) and  
618 viceversa (b,c). Scale bar = 15  $\mu\text{m}$  (a), 8  $\mu\text{m}$  (b,c), 10  $\mu\text{m}$  (d,e) or 50  $\mu\text{m}$  (f).

619 **Figure 5:** Testis sections of gilthead seabream at resting stage of a 1 year old fish untreated  
620 (C) (a, 5 days; d,f,g 3 days) or treated (b,c, 100 mg Cim/kg body mass, BM, 5 days; e, 200  
621 mg Cim/kg BM 3 days) immunostained with anti-BrdU serum (a,b,c) or subjected to  
622 TUNEL (d,e,f,g). Positive control of TUNEL was performed treating the section with  
623 DNase I before labelling (f) and negative control was performed omitting TdT enzyme in  
624 the reaction (g). Proliferative spermatogonia (asterisks) and Sertoli cells (arrows).  
625 Apoptotic Sertoli cells (white arrows) and spermatogonia (white arrowheads). Scale bar =  
626 25  $\mu\text{m}$  (a,b), 10  $\mu\text{m}$  (c) or 50  $\mu\text{m}$  (d,e,f,g).

627 **Figure 6:** *Dmrt1* gene expression in the gonad of 1 (a) and 2 (b) years old fish and serum  
628 levels of  $17\beta$ -estradiol ( $E_2$ ) (c,d) and 11-ketotestosterone (11KT) (e,f) in 1 (c,e) and 2 (d,f)  
629 years old fish at resting stage. Animals were injected with 0 (control), 50, 100 or 200 mg  
630 Cim/kg body mass (BM). Sampling was carried out after 3 and 5 days of Cim injection.  
631 The gene expression was analysed by real time PCR and data represent the means  $\pm$  S.E.M.  
632 of duplicate samples corresponding to four independent fish. The serum levels were  
633 analysed by ELISA and data represent the means  $\pm$  S.E.M. of duplicate samples  
634 corresponding to four independent fish. Asterisks indicate significant differences between  
635 treated and control groups (\* $P \leq 0.05$ ).

636 **Figure 7:** Expression levels of genes that code for steroidogenic-relevant molecules in the  
637 gonad of 1 (a,c,e,g) and 2 (b,d,f,h) years old gilthead seabream fish at resting stage. The  
638 expression of *star* (a,b), *cyp11a1* (c,d), *cyp11b1* (e,f) and *cyp19a1a* (g,h) in fish injected  
639 with 0 (control), 50, 100 or 200 mg Cim/kg body mass (BM) after 3 and 5 days of injection  
640 was analysed by real time PCR. Data represent the means  $\pm$  S.E.M. of duplicate samples  
641 corresponding to four independent fish. Asterisks indicate significant differences between  
642 treated and control groups (\* $P \leq 0.05$ ).  
643

644 **Table 1:** Gene accession numbers and primer sequences used for gene cloning and  
 645 expression analysis by real time PCR.  
 646

Gene	Accession	Name	Sequence (5'-3')	Use
<i>hrh1</i>		Hrh1-F1	CACACTGTTGGGAACCTCTA	cloning
		Hrh1-R1	GTGCTAGCCACGTAGTCCAT	
		Hrh1-F2	GATCGGTATCGCTCTGT	
		Hrh1-R2	AAAGCGGAAATCTGTGTCACA	
<i>hrh1</i>	LN875558	F	CTTGCCTCTGAACCTGGTGT	SYBR real time PCR
		R	AAATTGAGGCTGTGCTTGCC	
<i>hrh2</i>	KP728255	F	CCTAACACGCTTCACTCCGT	
		R	AGCTGCAGTTTTCTGTGGGA	
<i>hrh3</i>	KP728256	F	CTGTTTCAGCACACGGCTTC	
		R	GGCACACACGTACCACTACA	
<i>dmrt1</i>	AM493678	F	GATGGACAATCCCTGACACC	
		R	GGGTAGCGTGAAGGTTGGTA	
<i>star</i>	AM905934	F1	ACATCGGGAAGGTGTTCAAG	
		R1	TCTCTGCAGACACCTCATGG	
<i>cyp11a1</i>	FM159974.1	F	CGCTGCTGTGGACATTGTAT	
		R	CATCATGTCTCCCTGGCTTT	
<i>cyp11b1</i>	FP332145	F	GCTATCTTTGGACCCCATCA	
		R	CTTGACTGTGCCTTTCAGCA	
<i>cyp19a1a</i>	AF399824	F2	CAATGGAGAGGAAACCCTCA	
		R2	ATGCAGCTGAGTCCCTGTCT	
<i>rps18</i>	AM490061	F	AGGGTGTTGGCAGACGTTAC	
		R	CTTCTGCCTGTTGAGGAACC	

647  
 648  
 649

650 **Table 2.** Identification of the interest sequence in the teleost databases and their relation  
 651 with fish orthologs.

Predicted protein	Fish specie	Gene acc. number	Protein homology <sup>a</sup>	E value <sup>b</sup>
HRH1	<i>Sparus aurata</i>	CTQ87325.1		
	<i>Larimichthys crocea</i>	XP_010729625.1	93%	1e-52
	<i>Pundamilia nyererei</i>	XP_005724720.1	93%	7e-52
	<i>Dicentrarchus labrax</i>	CBN80867.1	93%	8e-52
	<i>Oreochromis niloticus</i>	XP_005459084.1	93%	1e-51
	<i>Fundulus heteroclitus</i>	XP_012724745.1	90%	3e-50
	<i>Oryzias latipes</i>	XP_011473142.1	90%	2e-49
	<i>Salmo salar</i>	XP_013989581.1	85%	1e-47

652 Percentage of homology (<sup>a</sup>) and E value (<sup>b</sup>) of the predicted proteins respect to the  
 653 CTQ87325.1 sequence.

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656

657 **Table 3:** Gonadosomatic index (GSI) and the gonad anti-BrdU immunostaining and  
 658 TUNEL staining of 0 (Control), 50, 100 and 200 mg Cim/kg body mass (BM) injected  
 659 gilthead seabream fish of 1 and 2 years old at 3 and 5 days post-injection (dpi).  
 660

		1 year old		2 years old	
		3 dpi	5 dpi	3 dpi	5 dpi
<b>GSI (%)</b>	Control	0.043 ± 0.017	0.041 ± 0.007	0.267 ± 0.066	0.279 ± 0.047
	50 mg Cim/kg BM	0.039 ± 0.003	0.046 ± 0.008	0.182 ± 0.022	0.135 ± 0.020*
	100 mg Cim/kg BM	0.053 ± 0.004	0.045 ± 0.013	0.295 ± 0.057	0.113 ± 0.010*
	200 mg Cim/kg BM	0.032 ± 0.003	0.040 ± 0.004	0.176 ± 0.012	0.169 ± 0.025*
<b>Anti-BrdU staining index</b>	Control	5.794 ± 0.702	4.251 ± 0.681	0.130 ± 0.050	1.715 ± 0.638
	50 mg Cim/kg BM	5.518 ± 0.934	7.152 ± 0.901*	0.288 ± 0.053	0.666 ± 0.121*
	100 mg Cim/kg BM	1.549 ± 0.3964*	9.874 ± 1.729*	0.146 ± 0.070	0.788 ± 0.155*
	200 mg Cim/kg BM	2.897 ± 0.657*	6.826 ± 0.859*	0.107 ± 0.044	0.796 ± 0.107*
<b>TUNEL staining index</b>	Control	1.125 ± 0.268	0.382 ± 0.138	1.7025 ± 0.681	0.2438 ± 0.040
	50 mg Cim/kg BM	0.760 ± 0.092	0.096 ± 0.026*	0.138 ± 0.051*	0.150 ± 0.032*
	100 mg Cim/kg BM	1.205 ± 0.397	0.143 ± 0.087	0.416 ± 0.171*	0.357 ± 0.069*
	200 mg Cim/kg BM	0.528 ± 0.109*	0.294 ± 0.065	0.130 ± 0.038*	0.164 ± 0.046*

661

662 Data represent means ± SEM of four independent fish/cimetidine (Cim) concentration and  
 663 dpi. Asterisks denote statistically significant differences between different concentrations of  
 664 Cim in the same time-groups according to Waller-Duncan post-hoc test ( $P \leq 0.05$ ).  
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Histological data published at  
Chaves-Pozo et al., 2009

Histological data published at  
Chaves-Pozo et al., 2005a;  
Liarte et al., 2007

SG PS R

SG S PS R

SG S PS TI

Sex change



Birth

One year old

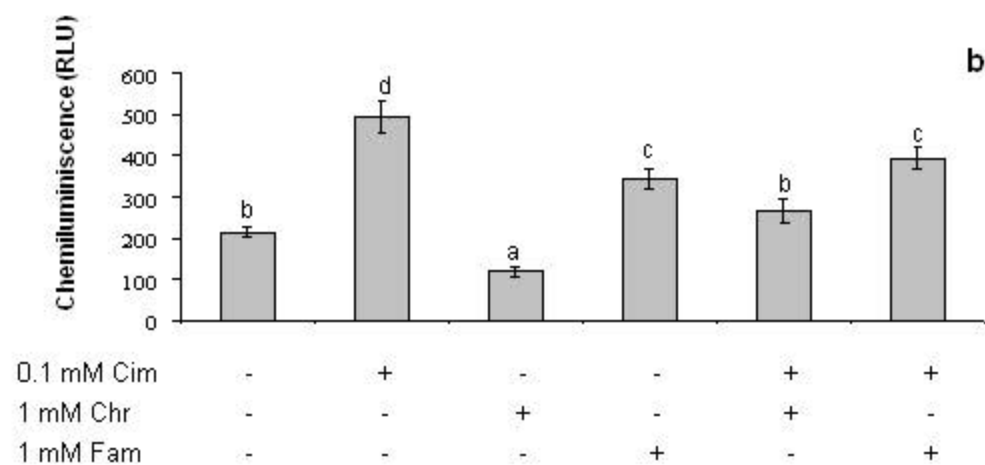
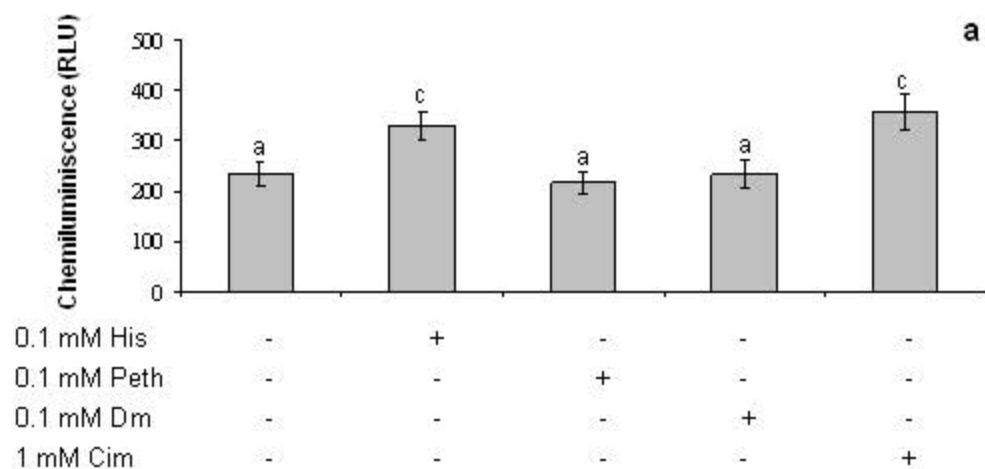
Two years old

Three years old

Four years old



Fig. 2





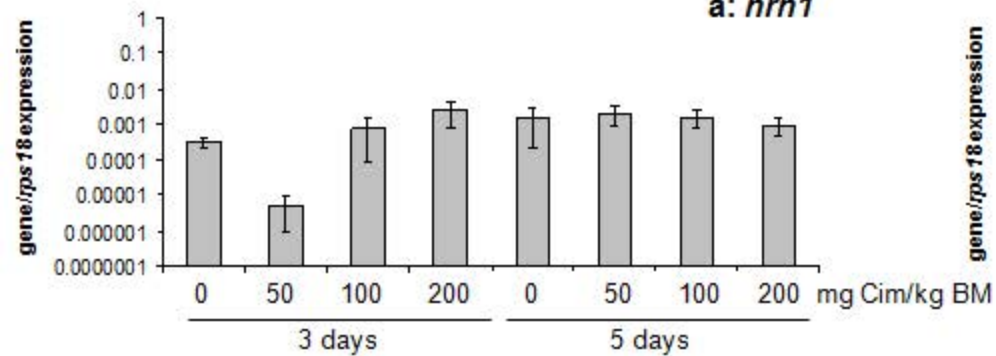
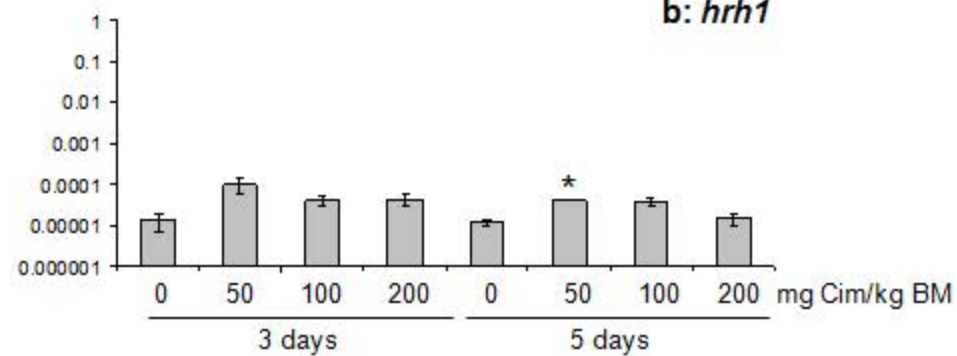
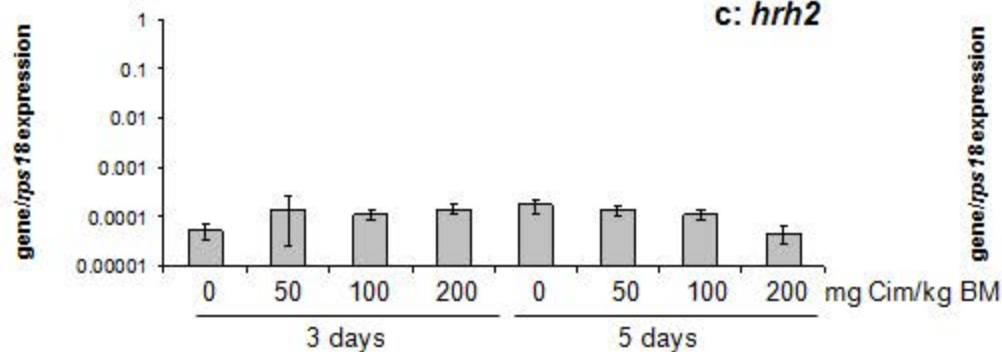
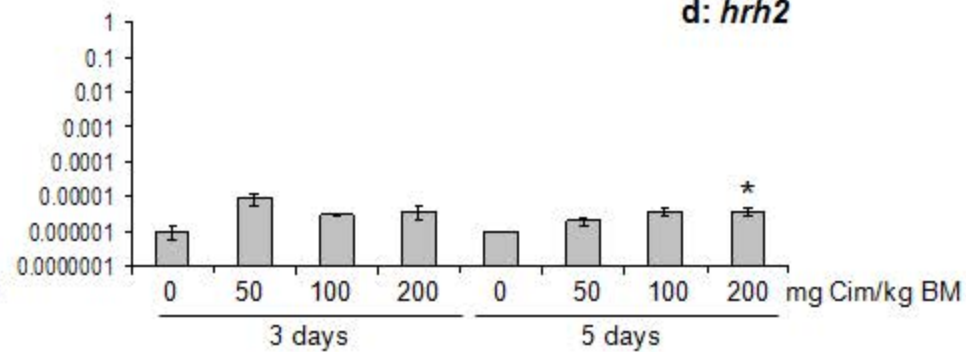
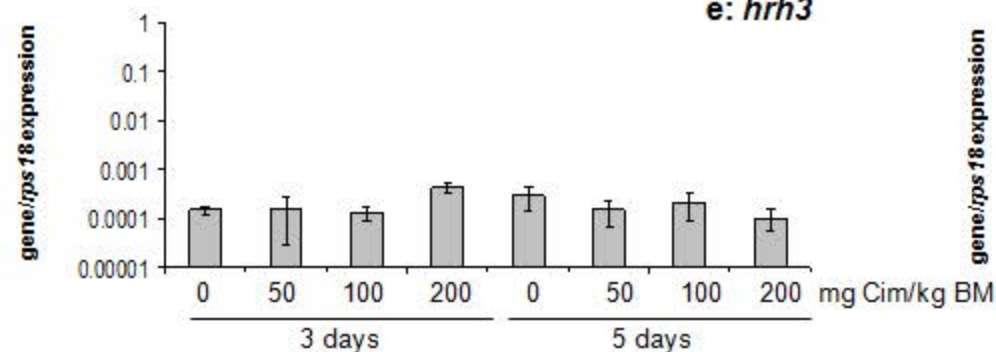
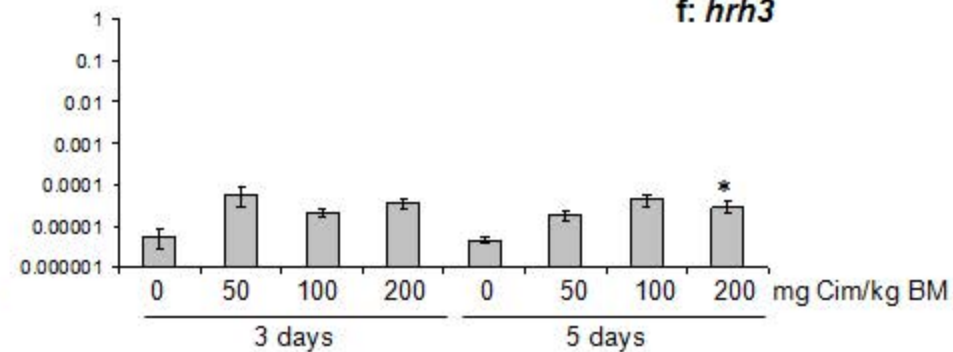
**1 year old****a: *hrh1*****2 years old****b: *hrh1*****c: *hrh2*****d: *hrh2*****e: *hrh3*****f: *hrh3***

Fig. 4

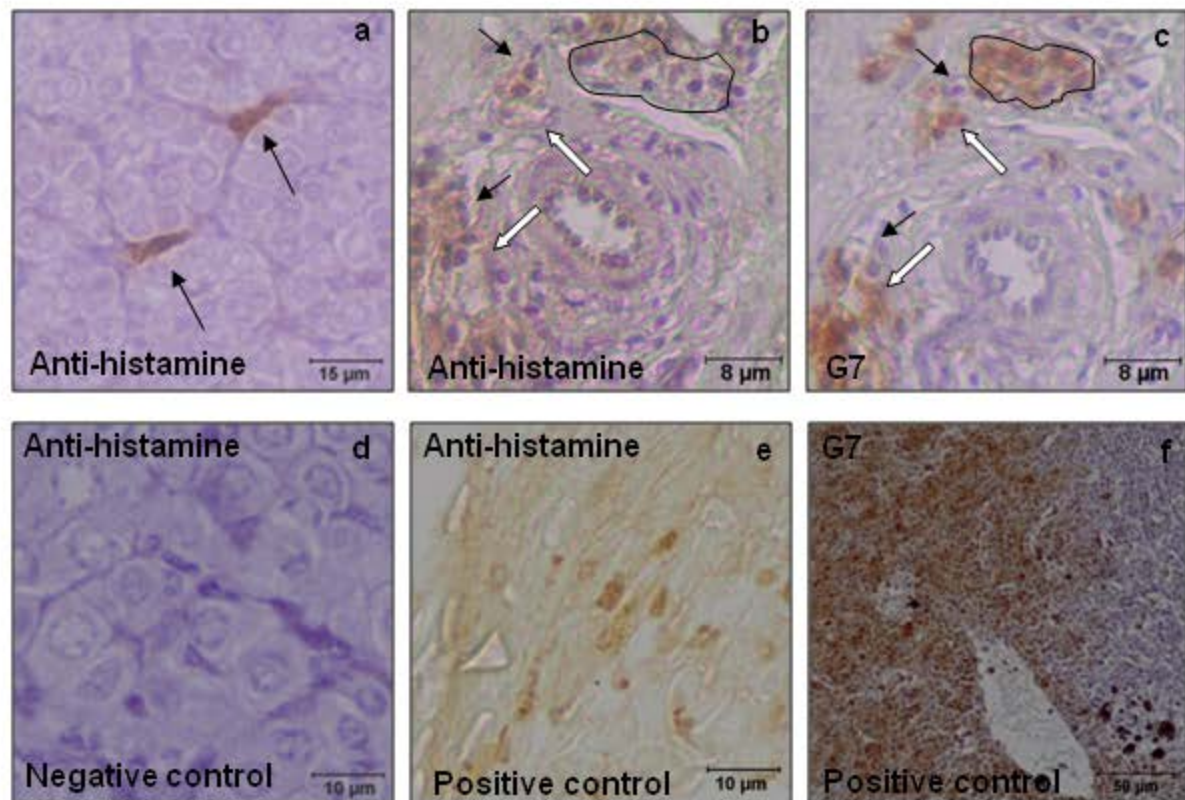
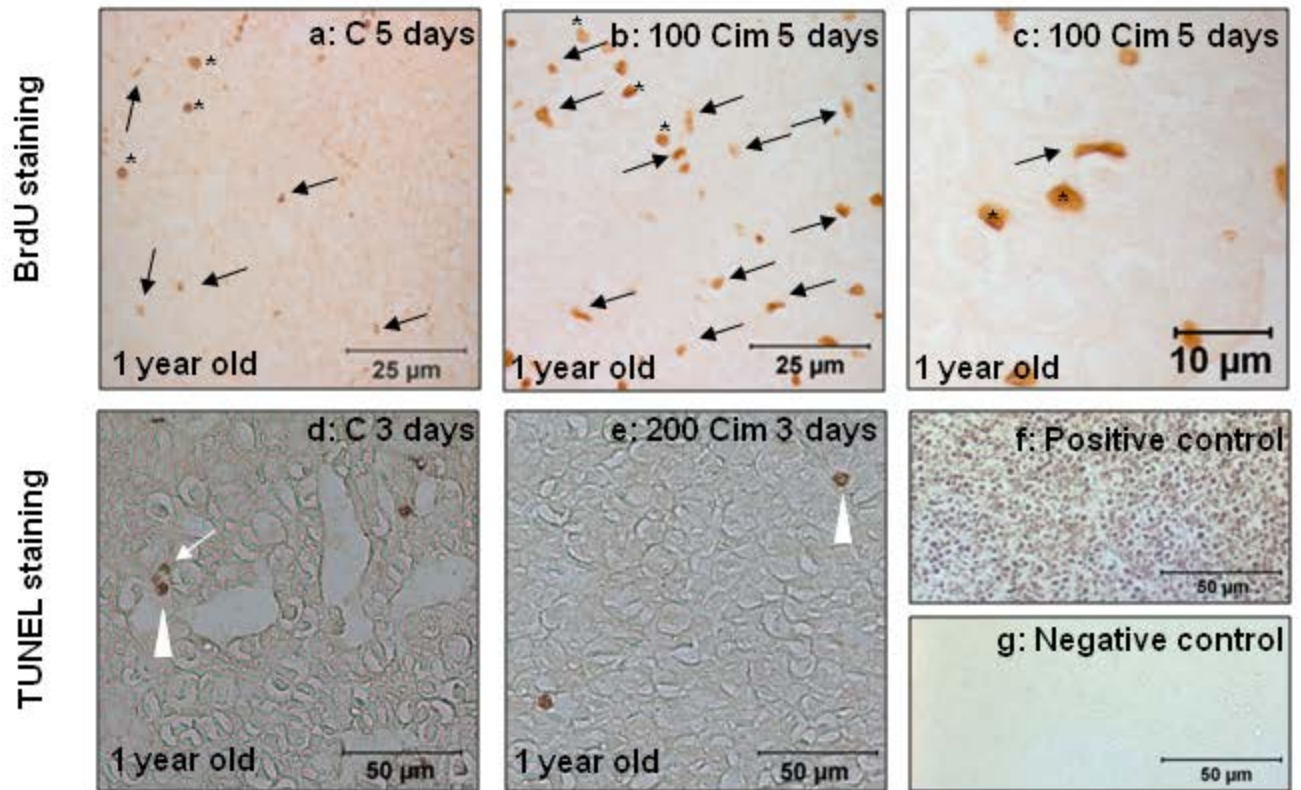
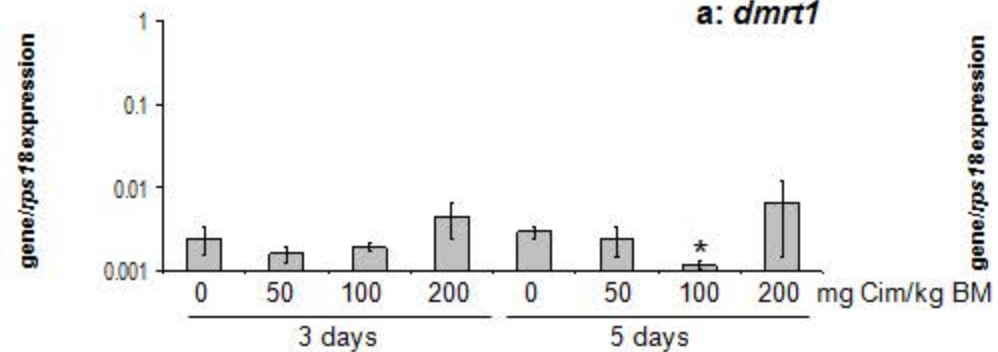
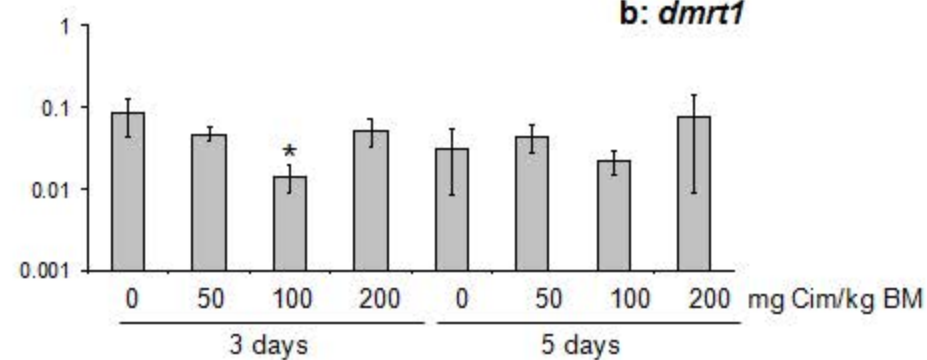
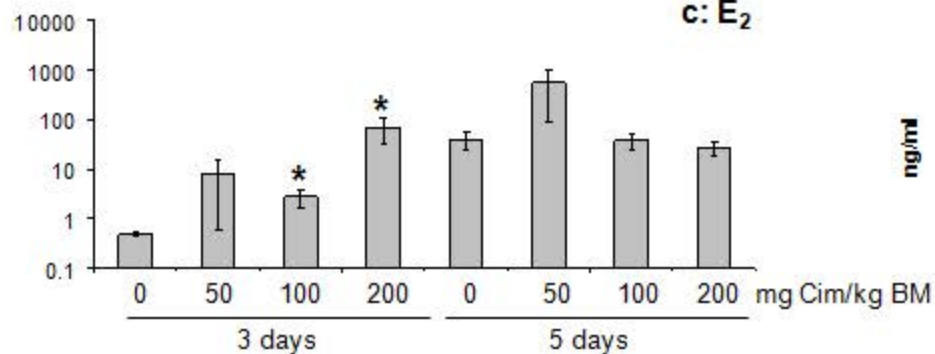
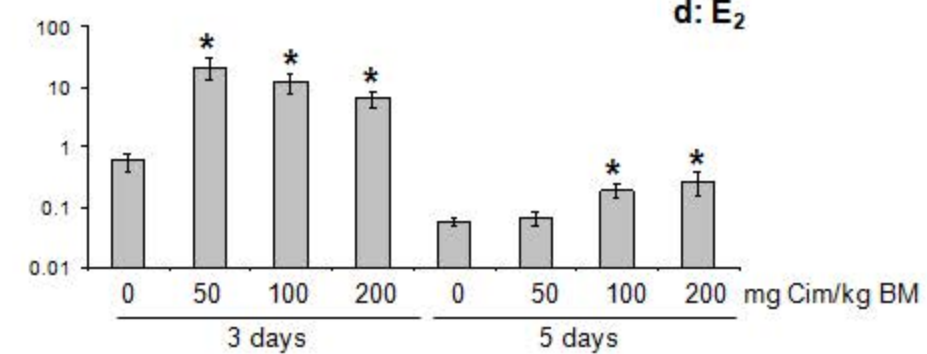
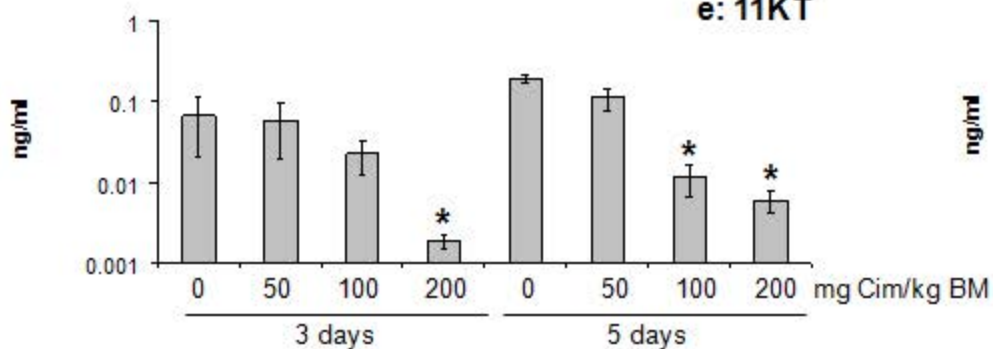
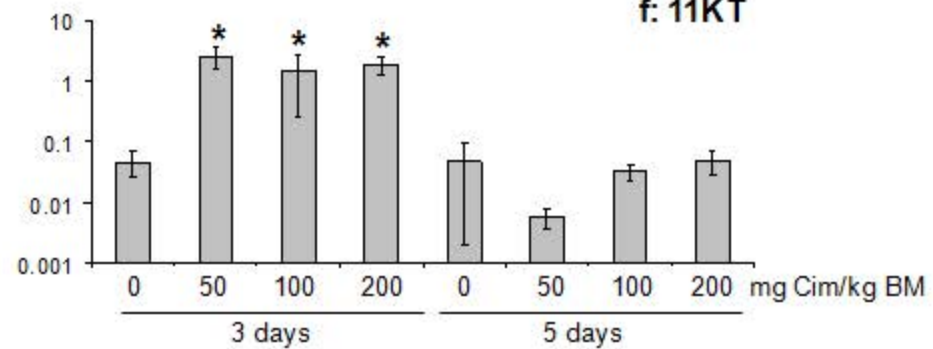
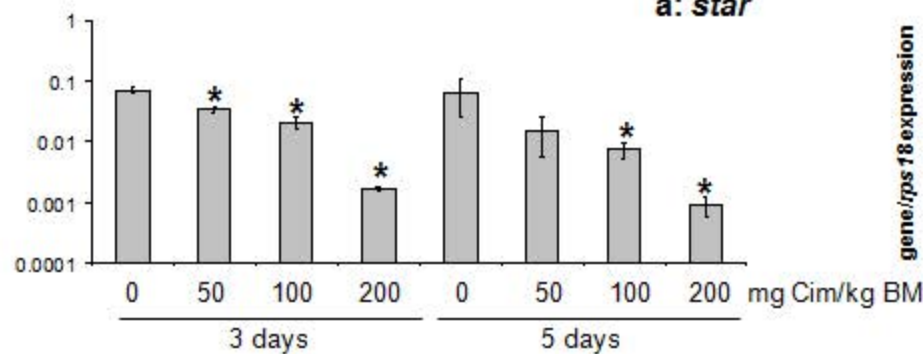
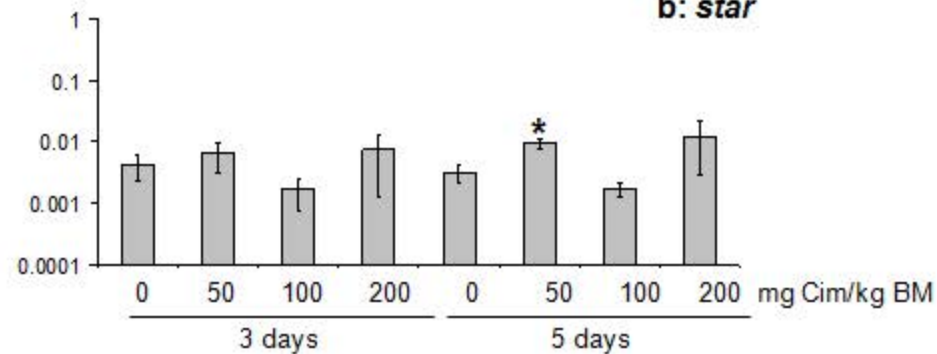
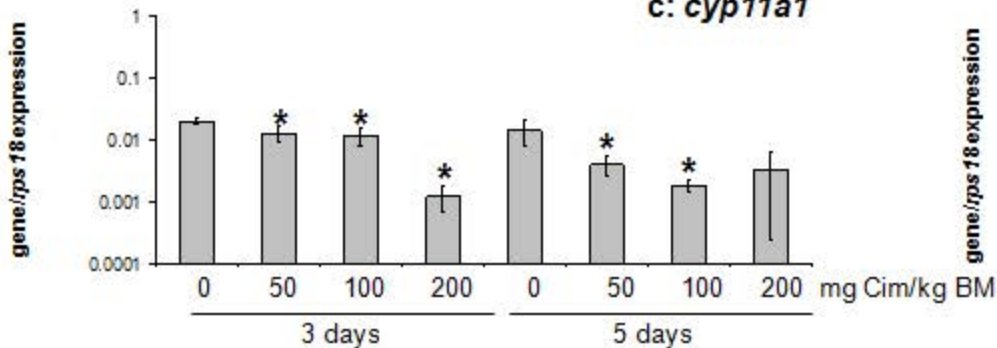
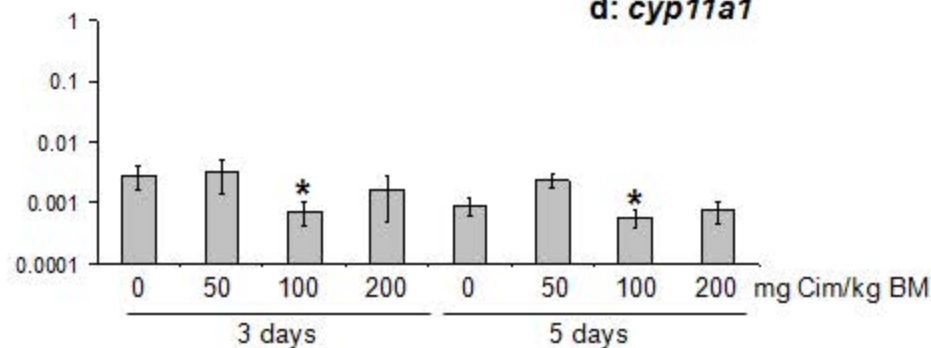
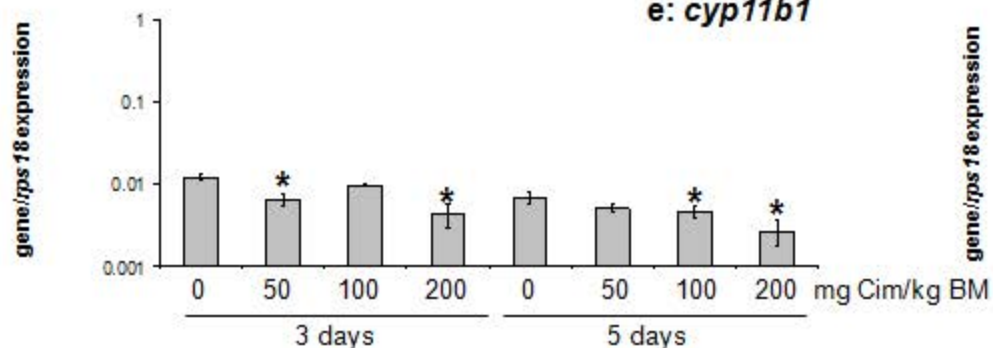
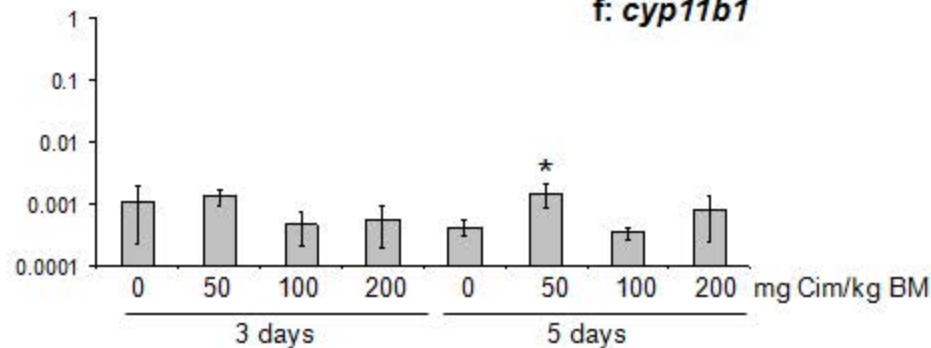
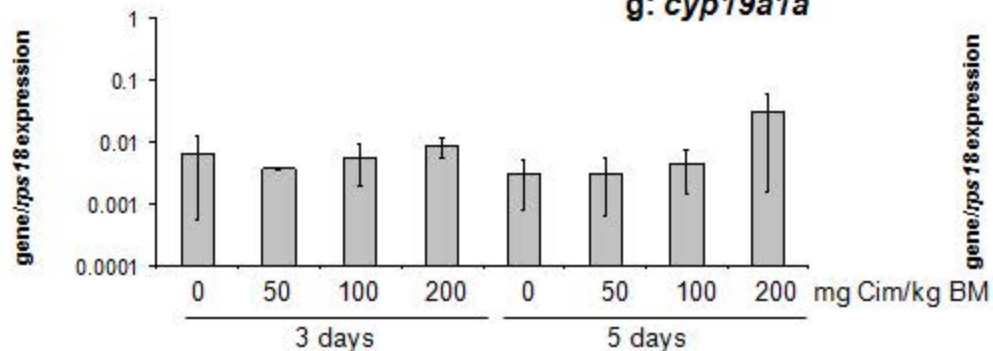
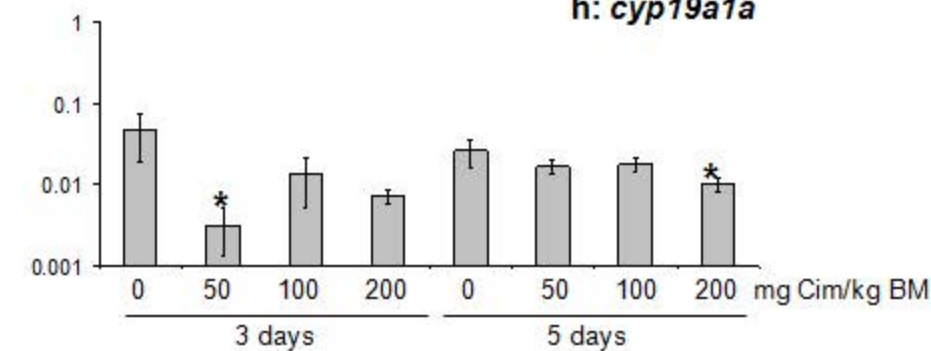


Fig. 5



**1 year old****a: *dmrt1*****2 years old****b: *dmrt1*****c: E<sub>2</sub>****d: E<sub>2</sub>****e: 11KT****f: 11KT**

**1 year old****a: star****2 years old****b: star****c: cyp11a1****d: cyp11a1****e: cyp11b1****f: cyp11b1****g: cyp19a1a****h: cyp19a1a**

**Highlights:**

1. Cimetidine acts through Hrh1 and Hrh2 in gilthead seabream leukocytes.
2. Histamine is present in the gonad of gilthead seabream.
3. The *hrh1*, *hrh2* and *hrh3* genes are expressed in the gonad of gilthead seabream.
4. Cimetidine affects cell renewal and steroidogenesis in gilthead seabream gonad.