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

Short title: Cimetidine disrupts the gonad of gilthead seabream

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Footnote[1]: The genetic nomenclature used in this manuscript follow the guidelines of Zebrafish Nomenclature Committee (ZNC) for fish genes and proteins and the HUGO Gene Nomenclature committee for mammalian genes and proteins.

Footnote[2]: 11-ketotestosterone (11KT), 17β-estradiol (E_2), 4% paraformaldehyde in PBS (PAF), 5-bromo-2'-deoxyuridine (BrdU), aromatase gene (*cyp19a1a*), body mass (BM), chlropheniramine (Chr), cholesterol side chain cleavage cytochrome P450 gene (*cyp11a1*), cimetidine (Cim), days post-injection (dpi), dimaprit (Dm), double sex-and mab3-related transcription factor 1 gene (*dmrt1*), famotidine (Fam), fetal bovine serum (FBS), gonad mass GM), gonadosomatic index (GSI), histamine receptors (HRs), *in situ* detection of DNA fragmentation (TUNEL), intraperitoneally (ip), monoclonal antibody (mAb), mAb specific to gilthead seabream acidophilic granulocytes (G7), phorbol myristate acetate (PMA), polyclonal antibody (pAb), pyridilethylamine (Peth), ribosomal protein S18 gene (*rps18*), steroid 11-beta-hydroxylase gene (*cyp11b1*), steroidogenic acute regulatory protein gene (*star*).

Abstract

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

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

1. Introduction

Cimetidine (Cim) is a pharmacological compound found in surface watercourses and recently in a coastal marine lagoon (Mar Menor, Spain) (Kolpin et al., 2002; Moreno-González et al., 2014; Ternes et al., 2001), Although no information exits on the presence of Cim in the open marine environment, it could be an emerging contaminant, as pharmaceuticals are consume all over the word, mainly in seas with little water exchange such as the Mediterranean Sea or nearly to the coast where fish farming occurred. In that sense, the assessment of how Cim can influence or disrupt marine living species physiology is important to define effective constrains measures. Measures that becomes highly important for commercial species with an important economic value for aquaculture such as the marine gilthead seabream (*Sparus aurata* L.). Cim has been reported in mammals as a potent antagonist of histamine H2-receptor (HRH2), but also as a partial agonist or as an inverse agonist of HRH2 depending on the ability of the cell to regulate the amount of HRH2 upon long term exposure to Cim (Smit et al., 1996; Takahashi et al., 2006; van der Goot and Timmerman, 2000). In mammals, Cim enhances tumor infiltrating lymphocytes responses, the antigen presenting capacity of dendritic cells and the interleukin 18 (IL18) production of monocytes, showing a potent anti-oxidative activity while also reducing pro-inflammatory cytokines production (Kubecova et al. 2011; Kubota et al., 2002; Takahashi et al., 2006). Moreover, Cim triggered the apoptosis of several testicular cell types and has been reported as a reproductive toxicant in male rats (Franca et al., 2000; Sasso-Cerri and Cerri, 2008). Recently, it has been reported that the dietary intake of Cim alters the non specific immunity in carps (Hosseinifard et al., 2013). Moreover, the exposure of adult

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

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

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

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

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

2. Materials and Methods

2.1. Animals and experimental design

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

fish were kept in 0.17 m^3 tanks with natural water temperature with a flow-through circuit, suitable aeration and filtration system and natural photoperiod.

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

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

2.2. Reactive oxygen intermediates production assay

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

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

2.3. Analysis of gene expression

Mammals and fish HRH1 gene sequences (*Danio rerio* NM_001042731.1; *Dicentrarchus labrax* CBN80867.1; *Bos taurus* NP_776508; *Equus caballus* NP_001075388; *Cricetulus griseus* ERE66926; *Mus musculus* AAK71654; *Pan troglodytes* NP_001107637; *Rattus 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). 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 ºC. 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

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

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

2.4. Light microscopy and immunocytochemical staining

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

μm. After dewaxing and rehydratation, some sections were stained with haematoxylin-eosin in order to determine the developmental state of each animal. Some serial sections fixed with Bouin's solution were subjected to an indirect immunocytochemical method using two antibodies: a polyclonal antibody (pAb) against histamine (Sigma) which has been previously used in the characterization of mast cells of gilthead seabream (Mulero et al., 2007) and a monoclonal Ab (mAb) specific to gilthead seabream acidophilic granulocytes (G7) (Sepulcre et al., 2002) which has been previously used in the characterization of testicular AGs (Chaves-Pozo et al., 2005b). Some sections fixed with PAF were subjected to an indirect immunocytochemical method using a mAb specific to BrdU (Becton Dickinson, San Jose, USA), at the optimal dilution of 1:100, that revealed the proliferative cells which have incorporated the BrdU, previously injected, during their DNA synthesis phase. Negative controls were done by omitting the first antiserum or in the case of the BrdU detection by using tissue sections from fish that had not been injected with BrdU. Some other sections fixed with PAF were subjected to *in situ* detection of DNA fragmentation (TUNEL) assay to identify apoptotic cells (*in situ* cell death detection kit; Roche, Basel, Switzerland) (Chaves-Pozo et al., 2007). Negative controls were processed in an identical manner, except that the TdT enzyme was omitted. Positive controls were also performed treating the sections with DNase I (3–3000 U/mL; Sigma) in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and BSA (1 mg/mL) for 10 min at room temperature to induce DNA strand breaks before labelling. Positive controls for anti-histamine immunostaining were performed using gut sections, in which histamine positive cells has been described in 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 17β-estradiol (E_2) 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 μL of serum in 1.3 mL of methanol (Panreac,
245 Barcelona, Spain). Then, methanol was evaporated at 37ºC and the steroids were
246 resuspended in 600 μL 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_3 (Sigma, St.
248 Louis, USA) and 0.1% albumin from bovine serum (Sigma, St. Louis, USA)]. Of this
249 solution, 50 μL were used for each ELISA reaction (2.5 μL of serum per reaction). A
250 standard curve from 6.13 x 10^{-4} 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_2 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 ºC using a Thermo Scientific Multiskan GO
257 plate reader. Standards and extracted plasma samples were run in duplicate and all the

readings were corrected with the blank and negative control. The lower detection limit for all the assays was 12.2 pg/mL. The inter-assay coefficients of variation at 50% of binding were 6.4% for E_2 (n=3) and 4.8% for 11KT (n=3). The intra-assay coefficients of variation (calculated from sample duplicates) were 8.2 ± 2.3% for E_2 and 6.9 ± 1.3% for 11KT assays. Details on cross-reactivity for specific antibodies were provided by the supplier (0.01% of anti-11KT reacts with T; and 0.1% of anti-E_2 reacts with T).

2.6. Calculation and statistics

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

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

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

were conducted using SPSS 12.0 (SPSS, Chicago, IL, USA). All data are presented as mean ± standard error to the mean (SEM).

3. Results

3.1. Identification of a partial sequence of hrh1 gene

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

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

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

cells for all compounds except for 1 mM Chr, which reduced cell viability to 94.88 ± 0.37%.

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

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

3.4. Histamine positive cells were present in the testis

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

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

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

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

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

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

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

Cim altered differentially the E_2 and 11KT serum levels of 1 and 2 years old gilthead seabream males. Regarding the E_2 serum levels, an increase was observed in fish of both ages, although the Cim concentration and the exposure time needed varied between both groups (at 3 dpi with 100 and 200 mg Cim/kg BM in 1 year old animals, Fig. 6c, and with all Cim concentration assayed in 2 years old animals and at 5 dpi with 100 and 200 mg Cim/Kg BM in 2 years old animals, Fig. 6d). In contrast, 11KT serum levels decreased in 1 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

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

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

In mammals and reptiles, histamine has been shown to modulate testicular 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

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

In mammals, histamine induces a dual concentration-dependent effect on Leydig cell steroidogenesis through HRH1 and HRH2 activation (Mondillo et al., 2009). In the gilthead seabream, Cim treatment triggered an increase in E_2 serum levels at both ages analysed while the 11KT levels were down-regulated in 1 year old fish and up-regulated in 2 years old fish. The effect on 11KT serum levels observed in 1 year old fish correlated with the down-regulation of gene expressions involved in androgen production, while *cyp19a1a* gene expression was unaltered. Moreover, despite of the changes observed in E_2 and 11KT serum levels in 2 years old fish, the expression the genes coding for steroidogenetic molecules scarcely varied in the gonad. Interestingly, differences between 1 and 2 years old fish were observed in all the processes studied, suggesting that the role of Cim in reproductive tissues depends on fish maturity, as one year old fish at resting stage

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

5. Conclusion

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

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8. Conflict of interest: The authors declare that they have no conflict of interest.

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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), pyridilethylamine (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 chlropheniramine (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 µg/mL). Data are
601 presented as mean ± S.E.M. of quadruplicate cultures and are representative of four
602 independent experiments. Letters determine significant differences between groups (*P ≤
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 fishs 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 ± S.E.M. of duplicate samples corresponding to four independent
609 fish. Asterisks indicate significant differences between treated and control groups (*P ≤
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

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

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

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

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

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
hrh1		Hrh1-F1	CACACTGTTGGGAACCTCTA	cloning
		Hrh1-R1	GTGCTAGCCACGTAGTCCAT	
		Hrh1-F2	GATCGGTATCGCTCTGT	
		Hrh1-R2	AAAGCGGAAATCTGTGTCACA	
hrh1	LN875558	F	CTTGCCTCTGAACCTGGTGT	SYBR real time PCR
		R	AAATTGAGGCTGTGCTTGCC	
hrh2	KP728255	F	CCTAACACGCTTCACTCCGT	
		R	AGCTGCAGTTTTCTGTGGGA	
hrh3	KP728256	F	CTGTTTCAGCACACGGCTTC	
		R	GGCACACACGTACCACTACA	
dmrt1	AM493678	F	GATGGACAATCCCTGACACC	
		R	GGGTAGCGTGAAGGTTGGTA	
star	AM905934	F1	ACATCGGGAAGGTGTTCAAG	
		R1	TCTCTGCAGACACCTCATGG	
cyp11a1	FM159974.1	F	CGCTGCTGTGGACATTGTAT	
		R	CATCATGTCTCCCTGGCTTT	
cyp11b1	FP332145	F	GCTATCTTTGGACCCCATCA	
		R	CTTGACTGTGCCTTTCAGCA	
cyp19a1a	AF399824	F2	CAATGGAGAGGAAACCCTCA	
		R2	ATGCAGCTGAGTCCCTGTCT	
rps18	AM490061	F	AGGGTGTTGGCAGACGTTAC	
		R	CTTCTGCCTGTTGAGGAACC	

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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[a]	E value[b]
HRH1	*Sparus aurata*	CTQ87325.1		
	Larimichthys crocea	XP 010729625.1	93%	1e-52
	Pundamilia nyererei	XP_005724720.1	93%	7e-52
	Dicentrarchus labrax	CBN80867.1	93%	8e-52
	Oreochromis niloticus	XP_005459084.1	93%	1e-51
	Fundulus heteroclitus	XP_012724745.1	90%	3e-50
	Oryzias latipes	XP_011473142.1	90%	2e-49
	Salmo salar	XP_013989581.1	85%	1e-47

652 Percentage of homology ([a]) and E value ([b]) 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**
GSI (%)	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*
Anti-BrdU staining index	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*
TUNEL staining index	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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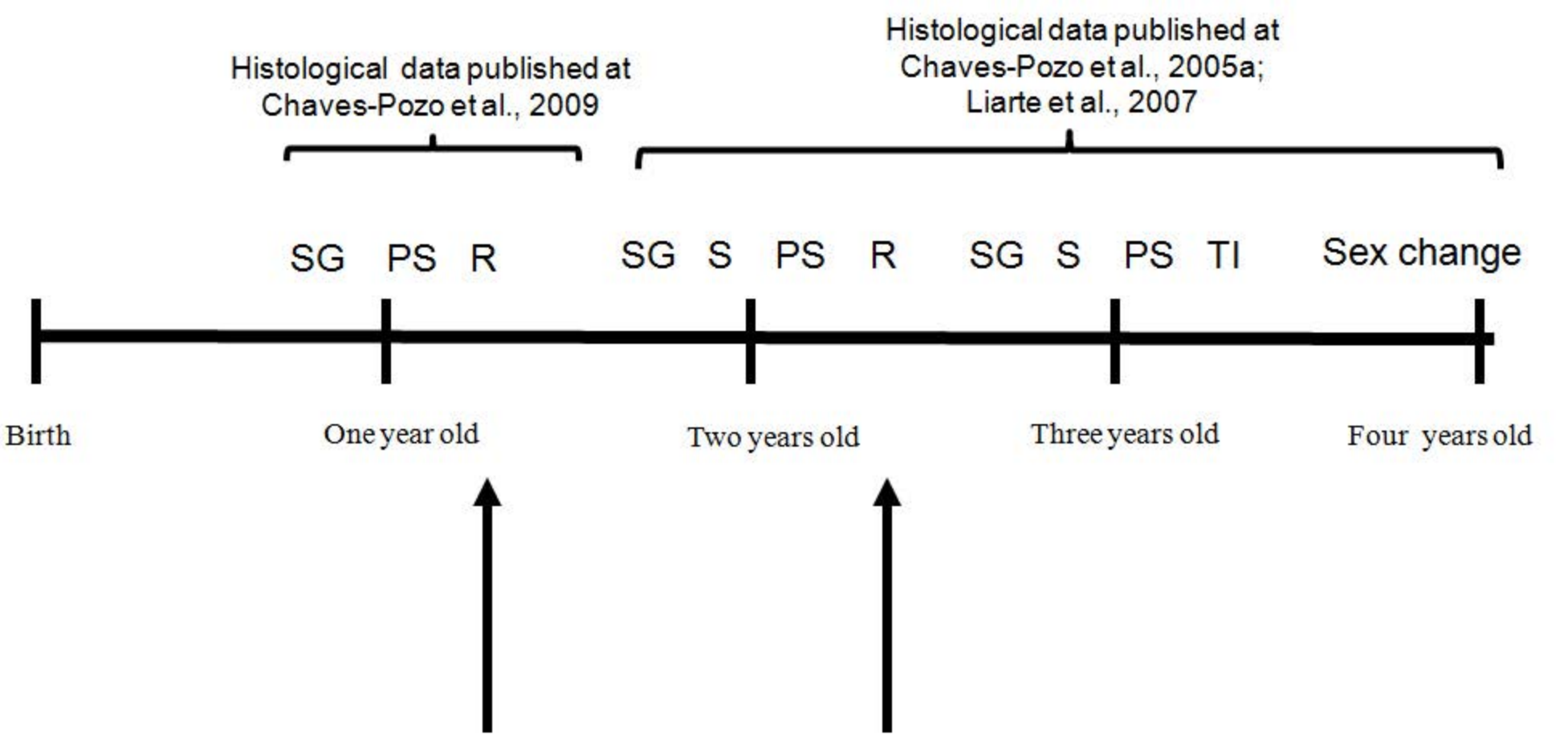
Histological data published at Chaves-Pozo et al., 2009
Histological data published at Chaves-Pozo et al., 2005a; Liarte et al., 2007
SG PS R
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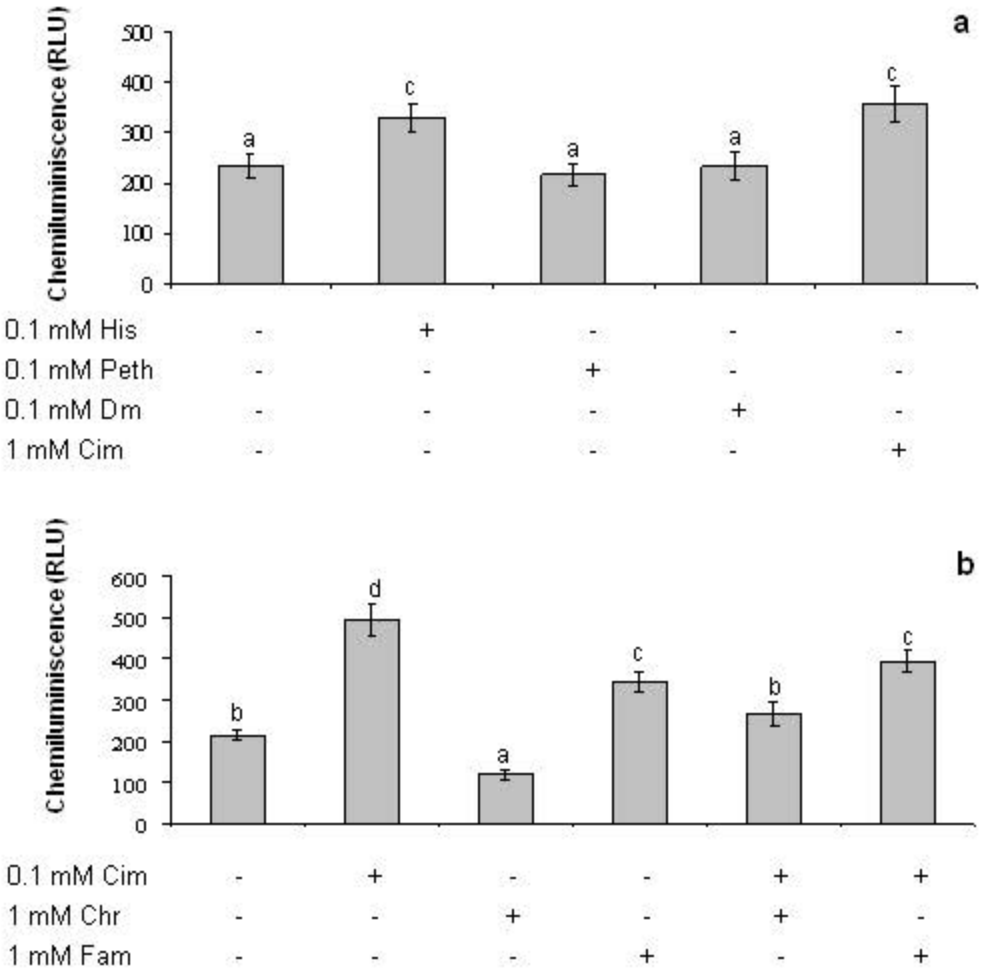
SG S PS TI
Sex change
Birth
One year old
Two years old
Three years old
Four years old

Fig. 2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Chemiluminiscence (RLU)
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c
0.1 mM His - + - - -
0.1 mM Peth - - + - -
0.1 mM Dm - - - + -
1 mM Cim - - - - +
b
Chemiluminiscence (RLU)
600
500
400
300
200
100
0
b
d
a
c
b
c
0.1 mM Cim - + - - + +
1 mM Chr - - + - + -
1 mM Fam - - - + - +

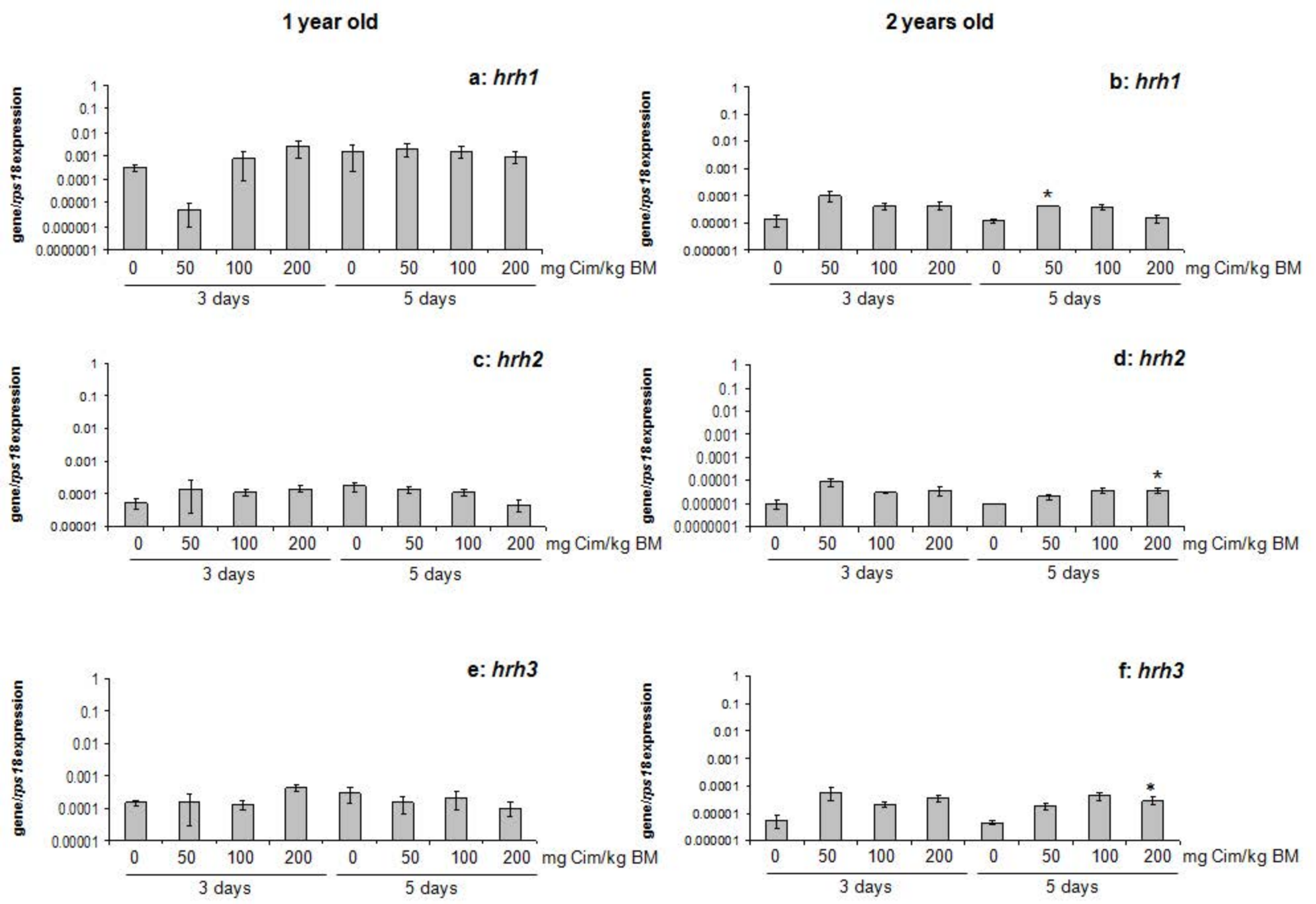

1 year old
2 years old
a: hrh1
b: hrh1
c: hrh2
d: hrh2
e: hrh3
f: hrh3
gene/rps18 expression
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0.0000001
0
50
100
200
mg Cim/kg BM
3 days
5 days
*

Fig. 4

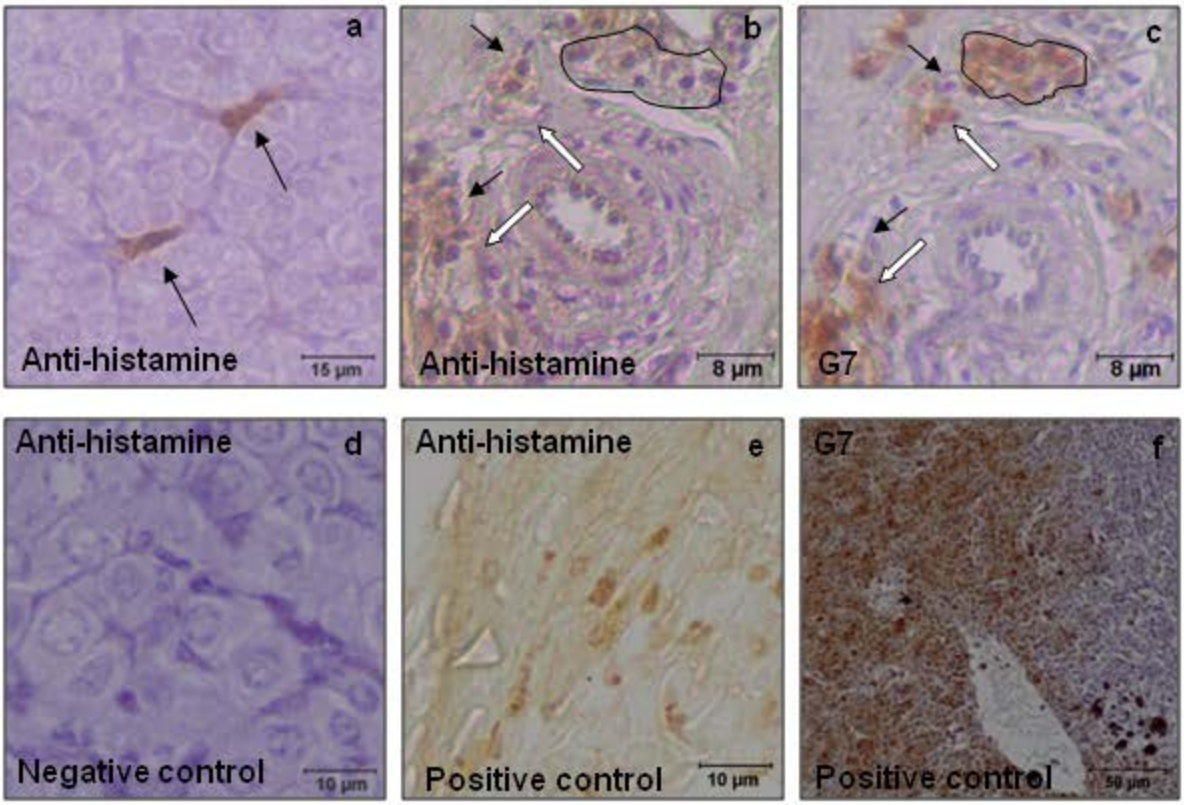
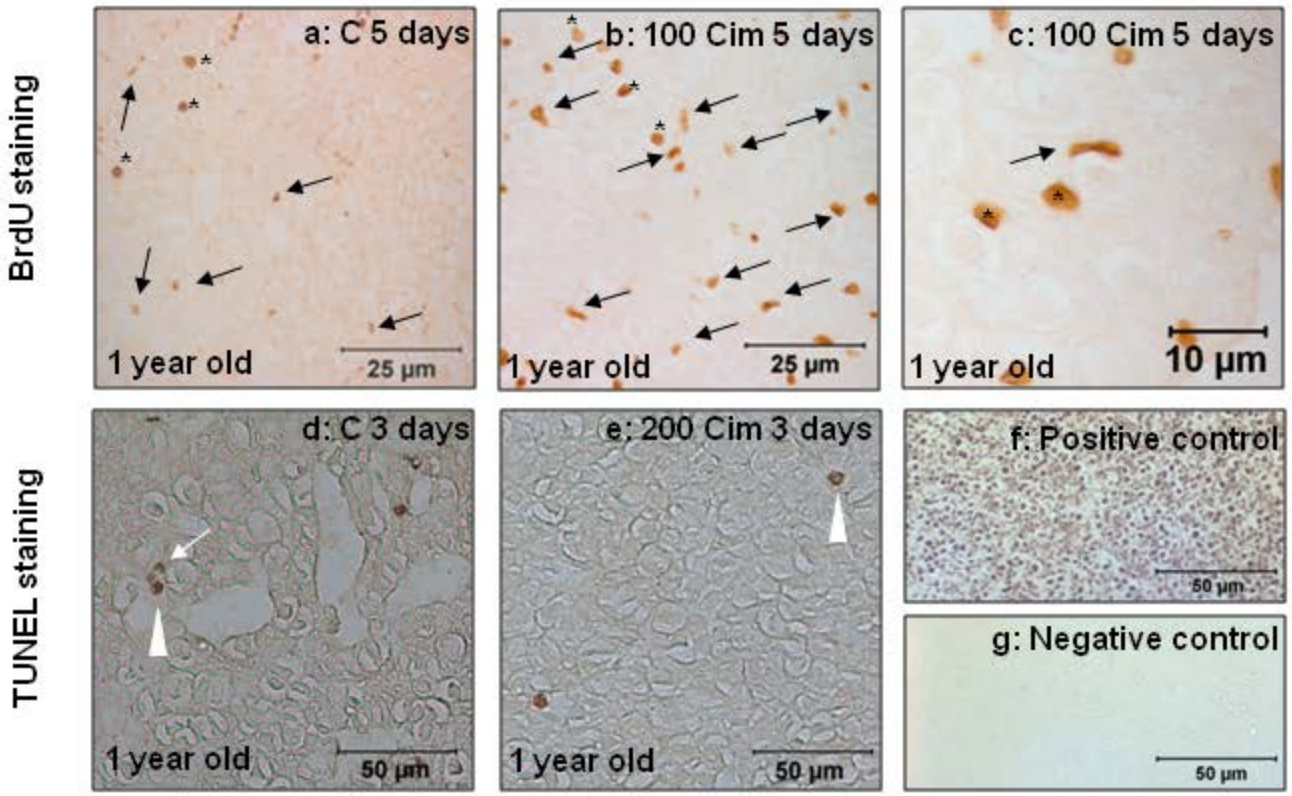
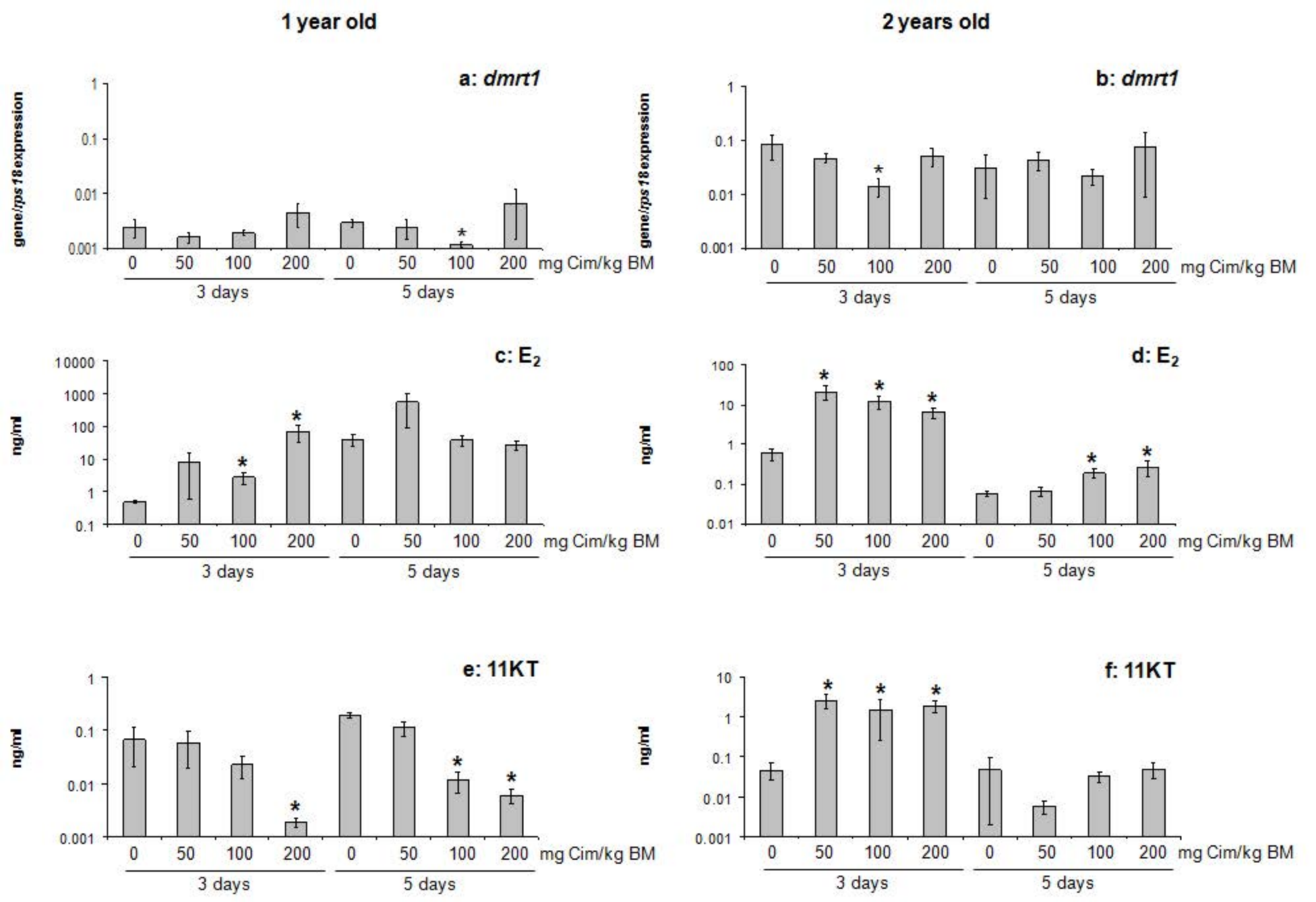
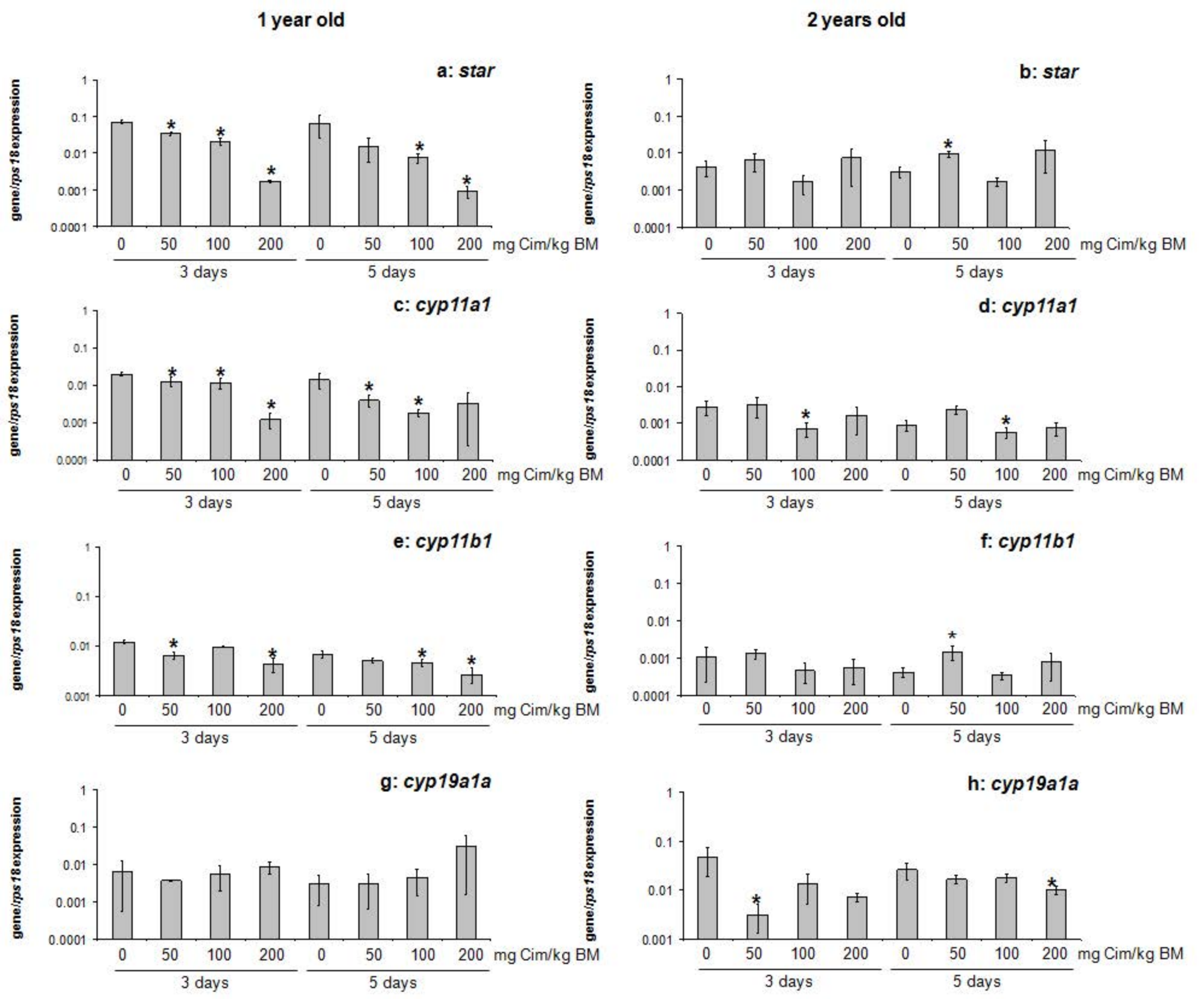

a
Anti-histamine
15 µm
b
Anti-histamine
8 µm
c
G7
8 µm
Anti-histamine
d
Negative control
10 µm
Anti-histamine
e
Positive control
10 µm
G7
f
Positive control
50 µm

Fig. 5


BrdU staining
a: C 5 days
1 year old
25 µm
b: 100 Cim 5 days
1 year old
25 µm
c: 100 Cim 5 days
1 year old
10 µm
TUNEL staining
d: C 3 days
1 year old
50 µm
e: 200 Cim 3 days
1 year old
50 µm
f: Positive control
50 µm
g: Negative control
50 µm


1 year old
2 years old
a: dmrt1
b: dmrt1
c: E2
d: E2
e: 11KT
f: 11KT
gene/rps18 expression
ng/ml
0
50
100
200
mg Cim/kg BM
3 days
5 days


1 year old
2 years old
a: star
b: star
c: cyp11a1
d: cyp11a1
e: cyp11b1
f: cyp11b1
g: cyp19a1a
h: cyp19a1a
gene/rps18 expression
1
0.1
0.01
0.001
0.0001
0
50
100
200
mg Cim/kg BM
3 days
5 days

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.