Epigenetics and phenotypic plasticity: histone modification in the European sea bass

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Abstract

Recent evidence shows that epigenetic modifications are involved in the long-lasting effects of elevated temperature during early development in fish. However, the evidence concerns DNA methylation, but other epigenetic modifications such as histone modifications have not been properly addressed. The objective of this study was to determine whether histone modifications could contribute to explain observed changes in gene expression in fish exposed to elevated temperature during initial stages of life. We used farmed European sea bass as the target species. We used chromatin immunoprecipitation (ChIP) to analyse two histone modifications: H3K4me3, associated with gene transcription activation, and H3K9me3, associated with gene transcription repression. Each modification was studied independently in three genomic features: the gene promoter, the region around the Transcription Start Site (TSS) and the immediate region downstream. The two genes considered were aq1 (aquaporin 1), which was not affected by temperature and was used as a control, and wisp1 (wnt< inducible signaling pathway protein 1), the expression of which was significantly downregulated by elevated temperature. The work carried out included all aspects of the technique including its optimization, to which a lot of efforts were devoted. Despite these efforts, results showed that, in accordance with what was expected based on gene expression data, no statistical differences were found in the two histone modifications in any of the three genomic regions studied in the aq1 gene. However, and in contrast to expectations, no differences were found either in the in the two histone modifications in any of the three genomic regions studied in the wisp gene. Further, H3K4me3 was enriched although in a non-statistically significant manner. We consider several factors to explain the lack of differences, including the limited sample size that resulted in large standard deviations or the genomic regions selected. Lack of direct relationship between histone modifications (for that matter, any type of epigenetic modification, including also DNA methylation) and gene expression patterns are not rare. In any case, our results show the complexity and intrinsic multifactorial nature of gene expression regulation in vertebrates.

Key words: epigenetic-modifications, histone, gene expression, early development, fish.
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2 Introduction

2.1 Histone modifications

In eukaryote cell nucleus, DNA is organized forming a high order structure called chromatin, in which 147 bp of DNA encircle an octamer of different histones, forming the nucleosomes. The histone octamer is constituted by two copies of H2A, H2B, H3 and H4 (Figure 1, Margueron & Reinberg, 2010).

Figure 1. General schematic depiction of the DNA organization forming nucleosomes and located in the cell nucleus. Different epigenetic modifications such as DNA methylation, histone post-translational modifications (PTM) and some histone variants are shown in the image (Margueron & Reinberg, 2010).

Chromatin not only allows to package the DNA, but also to control gene expression by epigenetic modifications. Generally, when chromatin is very compact gene expression is suppressed, because transcription factors and RNA polymerase are not able to bind to the DNA. Therefore, depending on the level of chromatin density, genes will be more or less expressed. The dynamic of the union between DNA and histones allows transcriptional regulation of genes. Histones have a globular domain involved in the association with other histones and they also have a positively charged domain which interacts with DNA phosphates. Post-translational histone modifications are present at lysine residues close to the N-terminal tail of histones 3 and 4. Both histone
modifications and histone variants take part in chromatin opening and closing (Fig. 1). All histone modifications present in a genome constitute the histone code model (Carlberg & Ferdinand, 2014).

Histones may be acetylated by enzymes called acetyl-transferases (HATs) or deacetylated by deacetylases (HDAC). The addition of acetyl group to a nitrogen atom of a lysine generally activates gene transcription, while deacetylation inhibits it. This gene control mechanism is linked to DNA methylation because this epigenetic modification attracts HDAC which is the enzyme responsible for removing acetyl group of histones and supressing gene transcription (Carlberg & Ferdinand, 2014). Histones can also be methylated. This modification may activate or suppress gene expression depending on the amino acid which has been methylated. In this case, one, two or three methyl group are added to a nitrogen atom of lysine. Moreover, there are also non canonical variants of histones which have differences in the amino acid sequence. Variant histones are less expressed than the canonical form, but affect chromatin remodelling and histone post-translational modifications (Biterge & Schneider, 2014).

In this study, two histone modifications were analysed. The first one is the trimethylation of lysine 4 on histone H3 (H3K4me3) which is related to gene expression activation because of its capability to block DNA methylation (Rose & Klose, 2014). The second one is the presence of trimethylation of lysine 9 on histone 3 (H3K9me3). In this case, H3K9me3 has an opposite effect due to the fact that it stimulates heterochromatin formation and gene expression silencing (Rose & Klose, 2014). Despite the fact that there are many post-translational modifications in different amino acids of histones, as acetylation, methylation, phosphorylation or ubiquitination (Carlberg & Ferdinand, 2014), only H3K4me3 and H3K9me3 were studied because of their known implication in epigenetic regulation of gene expression.

2.2 Environmental effects on histones

Most of the enzymes involved in chromatin remodelling are sensitive to environmental and metabolic changes. Thus, they work as sensors and are capable of modifying gene expression (Turner, 2009). Berger et al. (2009) proposed a model to explain the communication between the environment and the epigenetic status of cells (Fig. 2).
Environmental changes such as temperature are called epigenators, which are detected by cells. As a consequence, intracellular signalling pathways are activated, culminating in the activation of the epigenetic initiators. The initiators can be DNA binding proteins, non-coding RNA etc., responsible for local chromatin remodelling in response to the epigenator signal. Finally, there are also the epigenetic maintainers which sustain the epigenetic chromatin state across generations (Berger et al., 2009).

One example of temperature effects on histone modifications is vernalization in plants. Vernalization is the procedure by which plants acquire competence to flower when they are exposed to a low temperature period. This process relies on genetic and epigenetic basis. On one hand, the so-called “flowering locus C” (FLC) is a key gene in the vernalization pathway because it acts as a flowering repressor. Before vernalization, FLC expression is high. But in spring, when temperatures start to increase, FLC expression is repressed. FLC repression is related with the trimethylation of lysine 27 in histone H3 (H3K27me3), which is an epigenetic mark of repressed regions (Schmitz & Amasino, 2007).
2.3 Effects of temperature on fish

Temperature is one of the most important abiotic factors in fish development. Thermal conditions during early stages of development can lead to different phenotypes. Some studies with Senegalese sole have found that differences in temperature during embryonic stages may affect the gene expression and muscle development; an effect mediated by epigenetic mechanisms (Campos et al., 2013). Jonsson et al. (2014) in Atlantic salmon showed that fishes exposed to warm temperatures during larval stages presented higher growth rates than fishes subjected to ambient temperature. Sex ratio, which is a measure of the viability of a population, may be also affected by temperature. High temperature exposure during early development results in masculinization in European sea bass, which is associated with permanent changes in gene expression (Díaz & Piferrer, 2015). The histone variant H2A.Z has been found to mediate, at least partly, the plastic response of liver to seasonal temperature fluctuations in the carp (Simonet et al. 2013), suggesting that histone modifications and variants may play a key role in plastic responses of fish.

2.4 European sea bass

The European sea bass (Dicentrarchus labrax) is a teleost fish found in the north-eastern Atlantic Ocean and throughout the Mediterranean and Black seas (Tine et al., 2014). It is a gonochoristic species in which temperature and genetics drive equally sex determination (Navarro-Martín et al., 2009). There are plenty of studies about sex ratio shifts and the effects of temperature in the European sea bass. In all aquaculture populations, the males proportion increases resulting in male-biased sex ratio due to the exposure to high temperatures (21°C) during the thermo-sensitive period (TSP), between 0 and 60 days-post-fertilization (dpf) (Navarro-Martín et al., 2009). In aquaculture male-biased sex ratios are a problem because females are larger than males and reach sexual maturation later (Vandeputte et al., 2007). Navarro-Martín et al. (2011) have recently described the first epigenetic link between temperature and sex determination. It consists in the hypermethylation of the aromatase gene (cyp19a) promoter in one-year old juvenile females exposed at high temperature. The main function of aromatase is to convert androgens into estrogens, which are important in
ovaries differentiation in fishes (Guiguen et al., 2010). Therefore, this mechanism results in \textit{cyp19a} repression and in male development of the gonads.

There are different techniques to study epigenetic modifications. In this study, Chromatin ImmunoPrecipitation (ChIP) was used to analyse histone modification. ChIP is a technique to identify proteins associated with the DNA. This technique consists in incubation of chromatin fragments with an antibody for histones or specific DNA-binding proteins as transcription factors. In order to analyse ChIP data, it is needed to perform a quantification step after the IP as qPCR (ChIP-qPCR) or DNA sequencing (ChIP-seq). Here we used Native ChIP followed by Quantitative PCR.

3 Objectives

The objective of this study was to determine the presence of different histone modifications in a differentially expressed gene (DEG) in testis of adult European sea bass grown at different temperature conditions (low and high temperature) during the TSP. Therefore, we have focused on the influence of temperature in gene expression by histone modifications. Furthermore, one of the main goals of this study is to know whether epigenetic modifications arisen during early development are maintained along the years. For this, Chromatin ImmunoPrecipitation (ChIP) technique was carried out for two different histone modification (H3K4me3 and H3K9me3) in three specific gene regions (promotor, Transcription Start Site, Downstream). The expression of candidate genes was previously analysed by RT-qPCR in order to select DEGs.
4 Materials and methods

4.1 Animals and rearing conditions

Two-day-post hatch (dph) European sea bass larvae were obtained from the hatchery Base Viva de Sant Pere Pescador (Girona, Spain). 400,000 larvae were transported to our location in bags with the adequate oxygen and seawater. Fish were treated in agreement with the European Convention for the Protection of Animals used for experimental and Scientific Purposes (ETS Nu 123, 01/01/91). Our facilities are approved for animal experimentation by the Ministry of Agriculture and Fisheries (certificate number 08039-46-A) in accordance with Spanish law (Real Decreto 223 of March 1988) and the experimental protocol was approved by the Spanish National Research Council (CSIC) Ethics Committee. Animals were sacrificed by an overdose of 2-phenoxyethanol (2PE; 0.02 ml) followed by severing of the spinal cord.

4.2 Experimental setup

Two experimental groups were reared under two different temperature conditions during the first 60 days post fertilization (dpf). The “High Temperature” (HT) group grew until 7 dpf at 17 ºC and then the temperature increased 0.5ºC per day up to 21ºC. The “Low Temperature” (LT) group was subjected to a temperature of 17 ºC until 54 dpf and then temperature was raised 0.5ºC per day up to 21ºC. After the experimental period (68 dpf), both groups were exposed to natural temperature fluctuations. LT is the control group and temperature is the independent variable.

4.3 Sampling

After three years and twenty-seven days (1121 dpf), when sexual differentiation had finished, fish were sampled. Measures of length and body weight were carried out. We obtained sample of muscle and testis of each individual. The samples were frozen at the moment in liquid nitrogen and stored at -80 ºC. We were only able to use testis of three individuals of each group, not only because of other experiments using the same samples, but also because of the amount of sample necessary for the ChIP technique.
4.4 Selection of genes

The selection of DEGs was based on RNA-seq and methylation data of sea bass obtained in other GBR studies. Furthermore, the DEGs selected were compared with the work of Diaz and Piferrer (2015). Five genes differentially expressed and with differences in methylation between HT and LT groups were selected: *wisp1*, *junba*, *egr1*, *aql*, *scpep1*.

4.5 RT – qPCR

To verify the differential expression of these genes, RT – qPCR of mRNA samples was performed. We analysed four fish from LT and four fish from HT. Firstly, we did a DNase I treatment in order to eliminate any DNA sequence that may be there. For this treatment, 500 ng of RNA sample, 1 μl of 10X DNase I Reaction Buffer (200 mM Tris-HCl pH 8.4, 20 mM MgCl2, 500 mM KCl), 1 μl of DNase I, Amplification Grade (1U/μl) and MiliQ water to 10 μl were added to an RNase-free, 0.5 ml microcentrifuge tube on ice. Tubes were incubated for 15 min at room temperature. DNase I reaction was stopped by the addition of 1 μl of 25 mM EDTA solution and by heating for 10 min at 65 ºC. A reverse transcriptase-dependent conversion of RNA to cDNA was carried out after DNase I treatment. For the Retrotranscription, 100 ng of mRNA, 1 μl of random hexamers, 1 μl of 10 mM dNTP Mix (dATP, dGTP, dCTP and dTPP at neutral pH) and MiliQ water up to 13 μl were added to a nuclease-free microcentrifuge tube. The mixture was heated at 65 ºC for 5 minutes and incubated on ice for 1 minute. Finally, the following components were added: 4 μl of 5X First-Strand Buffer, 1 μl of 0.1M DTT, 1 μl of RNaseOUT™ Recombinant RNase Inhibitor (40 U/μl) and 1 μl of SuperScript™ III RT (200 U/μl). The tubes were incubated at 25 ºC for 5 minutes, at 50 ºC for 30 minutes and at 70 ºC for 15 minutes in order to inactivate the reaction. Finally, we performed qPCR using specific primers for the candidate genes. Primers were designed using the European sea bass genome (Tine et al, 2014), targeting only the exons of the genes and using the primer3plus program. We checked whether the sequences dimerized between themselves by the Oligos program. Primers efficiency was checked by using dilutions (1, 1/5, 1/10, 1/50, 1/100 and 1/500) of a pool of 20 μl of each cDNA sample. For the qPCR 2 μl of cDNA, 0.5 μl of each primer (10 μM), 2 μl of EvaGreen and 5 μl of H2O MiliQ were added. Triplicates and negative controls
(without cDNA) were used. Furthermore, two genes (fau and ef1a) with known expression that does not change between groups were used as reference genes. RT-qPCR reactions were carried out using an ABI 7900HT (Applied Biosystems) and were run with the following cycling conditions: UDG decontamination at 50ºC for 2 minutes, initial activation at 95 ºC for 10 minutes and 40 cycles of denaturation at 95 ºC for 15 seconds and annealing /extension at 60 ºC for 60 seconds. Finally, we added a dissociation step, which consisted of 15 seconds incubations at different temperatures (95 ºC, 60 ºC and 95 ºC). Data were analysed by Relative Quantification by Comparative Ct method (2ΔΔCT).

4.6 ChIP procedure

In order to study histone modifications, we carried out assays of Chromatin immunoprecipitation (ChIP) followed by Quantitative PCR (qPCR). During the ChIP procedure all steps were done on ice or at 4 ºC to avoid chromatin degradation. Before carrying out ChIP, all the protocol steps were optimized for our experiment (Annex 1). All buffers used are specified in Annex 1. Testis of each sample was homogenised with a homogenization buffer or Buffer H (0.25 M sucrose, 0.02 M Tris, 3 mM CaCl2 and protease inhibitor cocktail) and using a glass pistil. Buffer H was added up to 1 ml and a centrifugation step was carried out at 1000g for 10 min at 4 ºC. The supernatant was discarded. We did three washes with 500 µl of Buffer H followed by centrifugation steps (1000 g for 10 min at 4 ºC) in order to extract nuclei, where chromatin is located. Finally, the pellet was resuspended in 500 µl of Buffer H. DNA purification with phenol – chloroform – isoamyl alcohol method (PCI; 25:24:1) was employed to estimate the amount of chromatin present in each sample. Chromatin was sheared by enzymatic digestion with Micrococcal nuclease (Mnase). Shearing was performed by 1 Unit of enzyme for each microgram of chromatin during 20 minutes at 37 ºC. A 1/50 dilution of the enzyme was needed to have a concentration of 40 U/µl. In a final volume of 500 µl we added to the initial sample 50µl of BSA (10 mg/ml), 10 µl of 10X Micrococcal nuclease Buffer and MiliQ water. After the incubation a buffer containing EDTA (final concentration of 5 mM) was added and the sample was incubated on ice for 5 minutes to stop the enzymatic reaction. Finally, the tubes were centrifuged at 8000 g for 5 minutes at 4 ºC and the supernatant was recollected.
We used 20 µg of chromatin for each Immunoprecipitation (IP) with antibodies for two histone modifications: H3K4me3 (ab8580; Abcam) and H3K9me3 (Ab8898; Abcam). It was also necessary to perform another IP with an unspecific antibody IgG (ab172730; Abcam), which was the negative control. Finally, 20 µg more were used as Input sample (positive control). The direct method has been used for the IP, in which Dynabeads® and antibodies are incubated during 6 hours at 4 ºC (prebinding). In this step, we added for each IP: 4 µg of antibody, 30 µl of Dynabeads® and RIPA-150 Buffer (50 mM Tris-HCl pH 8, 0.15 M NaCl pH 8, 1mM EDTA pH 8, 0.1 % SDS, 1% Triton X-100, 0.1 % sodium deoxycholate) up to 500 µl. After prebinding, the tubes were placed on a magnetic rack to discard the supernatant and samples were added. An overnight incubation on rocker at 4 ºC was carried out. Several washes were performed to discard the chromatin that was not bound to the antibodies and keep the fraction that was bound. The wash steps were performed with 800 µl of the corresponding buffer followed by an incubation for 5 minutes at 4º C. After each wash the supernatant was discarded using a magnetic stand. The following washes were carried out: one time with RIPA-150 Buffer, two times with RIPA-500 Buffer (50 mM Tris-HCl pH 8, 0.5 M NaCl pH 8, 1mM EDTA pH 8, 0.1 % SDS, 1% Triton X-100, 0.1 % sodium deoxycholate), two times with RIPA-LiCl Buffer (50 mM Tris-HCl pH 8, 1mM EDTA pH 8, 1 % Nonidet P-40, 0.7 % sodium deoxycholate, 0.5 M LiCl2) and two times with 1X TE Buffer pH 8 (10 mM Tris-HCl pH 8, 1 mM EDTA pH8) without incubation step. Finally, IP elution buffer (10 mM Tris-HCl pH 8, 0.3 M NaCl pH 8, 5mM EDTA pH 8, 0.5 % SDS) was added to separate the antibody from the chromatin. 2 µl of RNase A were added to the IP and the mixture was incubated at 65 ºC for 4 hours. Finally, the supernatant was kept and the DNA was purified with phenol – chloroform – isoamyl alcohol method in order to perform a qPCR. In this step we added the Input sample.

For the qPCR we designed primers targeting ~100 bp around three regions (~2000bp upstream the Transcription Start Site or Promoters, Transcription Start Site or TSS and ~2000bp Downstream of the Transcription Start Site or DS) of two genes: wnt1 inducible signaling pathway protein 1 (wisp1) and aquaporin 1 (aq1). The first gene was less expressed in HT and the second one was equally expressed in both groups. The design of primers was carried out using the sea bass genome and the primer3plus program. Primers efficiency was checked by using dilutions (1, 1/5, 1/10, 1/50, 1/100 and 1/500) of a pool of 20 µl of each DNA sample used in the experiment. To perform
the qPCR, 2 µl of chromatin, 0.5 µl of each primer (10 µM), 2 µl of EvaGreen and 5 µl of H2O MiliQ were added. Triplicates and negative controls (without DNA) were used. QPCR reactions were carried out using an ABI 7900HT (Applied Byosistems) and were run with the following cycling conditions: 50ºC for 2 minutes, 95 ºC for 10 minutes and 40 cycles at 95 ºC for 15 seconds and 60 ºC for 60 seconds. Finally, we added a dissociation step, which consisted of 15 seconds incubations at different temperatures (95 ºC, 60 ºC and 95 ºC). In this case, the same Comparative Ct method was used.

4.7 Statistical data analysis

For both statistical analysis (RT-qPCR and ChIP), the Comparative Ct method was used. For the analysis of expression measured by RT-qPCR, the difference between two groups (LT and HT) is given by Delta delta Ct (ΔΔCt), also called Fold Change (FC):

\[ ΔΔCt = ΔCt (sample \ of \ interest) - ΔCt (reference \ simple) \]

In our case:

\[ ΔΔCt = (CtLT - CtHT) - (Control \ gene \ LT - Control \ gene \ HT) \]

The ΔCt value describes the difference between the Ct value of the target gene and the Ct value of the endogenous reference gene. We then, calculated the ΔΔCt, which describes the difference between the average ΔCt value of the HT group and the average ΔCt value of the LT group, the reference one.

Furthermore, the efficiency of each target and endogenous gene was calculated and we checked that the efficiencies were similar and close to 2, which is the 100 % efficiency. The indicator used for the efficiency was the slope of the standard curve. A 100% of efficiency means that the PCR product doubles during each cycle and it is achieved when the slope is -3.322. To calculate the efficiency, we used the following equation:

\[ E = (10^{1/slope} - 1) \times 100 \]

The error was calculated using the standard propagation of error method. Significance
was checked by t-student test, which compares LT and HT groups. Levene’s test was done to assess the homogeneity equality of variances and normality of residuals was also checked by Shapiro-Wilk test. For those data that did not complied with equality of variances (p-value< 0.05 in Levene’s test) or normality (p-value<0.05 in Shapiro-Wilk test), a non-parametric test (Kruskal-Wallis test) was performed.

In the case of the ChIP statistical analysis, there are no reference genes, but the Input must be considered as the reference value (positive control). Input amplification is higher (lower Ct values) than IPs (higher Ct values), because in the input sample there is all the chromatin, not only the immunoprecipitated one. The difference between two groups is given by:

$$
\Delta \Delta Ct = (Ct \text{ IP LT} – Ct \text{ Input LT}) – (Ct \text{ IP HT} – Ct \text{ Input HT})
$$

In this case, the efficiency was also calculated with the same method. Calculation of standard error was done using the standard propagation of error method. T-student test was also done in order to determine the significance of the results through p-value. Levene’s test and Shapiro-Wilk test were done to check homogeneity of variances and normality of residuals respectively. For the non-homogeneous or non-normal data, Kruskal-Wallis test was done.
5 Results

5.1 ChIP optimization

Two different ways of nuclei purification of sea bass testis are shown in Figure 3. Sample 1 and 2 belong to the same fish, but nuclei were extracted using different ways. Sample 1 was treated with the standard cleaning described in ChIP protocol (Annex 1), whereas sample 2 nuclei were purified with the standard and with a further cleaning of the nuclei through sucrose gradient. It seems that sample 2 has less DNA concentration than sample 2, because of the band intensity and the DNA concentration measured by Nanodrop. DNA concentration value was higher in the sample 1.

Figure 3. 1.5 % w/v agarose gel, stained with SYBR safe where there are two samples of purified nuclei in two different ways. Both samples have been treated with Buffer H, but one of them (2) has been submitted to an optional wash using different buffers with different sucrose concentrations. Band 2 is smaller than band 1, due to the fact that it has a minor DNA concentration.

Figure 4 revealed the chromatin fragments obtained from the same sample as a consequence of sonication at different times. A size of 200-1000 bp is needed for the chromatin fragments. In the test done at 30 min-sonication (Fig. 4A) the fragments are still too large. In figure 4B shearing is observed in a range of 120 minutes. The non-fragmented sample (0) and the one sheared for 20 minutes (20) have quite large DNA fragments. As for the sample sonicated for 40 minutes (40), the fragments are smaller. In contrast, the one sheared for 60 minutes show bigger fragments. The sonicated sample for 80 minutes was lost by mistake during the DNA extraction. Finally, samples sheared for 100 and 120 minutes are those with smaller fragments. The inconsistencies in fragment length obtained by sonication led us to test other fragmentation methods.
Figure 5 shows the results obtained from enzymatic chromatin digestions with Micrococcal nuclease at different concentration and at different times. Firstly, the time for the enzymatic digestion was optimized (Fig. 5A). Thus, the enzymatic chromatin digestions with 1U of Micrococcal nuclease per µg of chromatin (1U/ µg) at different times (5, 10, 15 and 20 minutes) were performed. DNA concentrations obtained are low due to the small quantity of sample used for the DNA extractions and to the amount of DNA present in the sample. However, we decided to use 20 minutes of chromatin shearing to achieve the fragments length desired (200 - 1000 bp). Optimization of Mnase concentration necessary to digest 1 µg of chromatin is shown in Figure 5B. We did digestions of the same sample for 15 minutes at different concentrations: 1U, 2U, 3U, 4U and 5U/µg. In non-digested sample (0) there are fragments of DNA of all sizes. When concentration of the enzyme increases, the fragments are smaller. A concentration of 3U/ µg is enough to achieve the size of fragments desired.
5.2 Differentially expressed genes

Our RT-qPCR results show the expression of candidate genes (*wisp*, *egr1*, *aq1*, *junba* and *scpep1*) in European sea bass grown at low and at high temperature (Fig. 6). Gene expression is represented as Fold Change (FC), which is not an absolute value, but relative to LT group. Thus, FC compares expression between the two groups. We found a lower expression in the HT group (negative FC value) in *wisp* (-2.8156), *junba* (-0.2336) and *scpep1* (-0.4770) genes. Meanwhile, the expression was higher (positive FC value) for *egr1* (0.2458) and *aq1* (0.0209) genes in HT groups. Nevertheless, the only gene with a significant differential expression between the two conditions is *wisp1* (p-value = 0.0329), which is less expressed in HT. No significant differences were found for the rest of genes (p-value > 0.05). We selected *wisp* as the differentially expressed gene, since it showed the highest FC, and *aq1* as the control gene, because it showed the least FC, for the ChIP experiment. P-values of the statistical tests performed are shown in Annex 2.

![Figure 6. RT-qPCR analysis of different genes (previously known to have differences in expression) from testis of sea bass treated at high (HT) and low temperature (LT). In the graph the fold change of the HT group in comparison to the LT group as the reference group is shown, as well as the lower and upper confidence interval.](image)
5.3 Histone modifications

The results obtained in ChIP-qPCR analysis are shown in Figure 7. Delta Delta Ct values (ddCt) is presented for three regions (Promoter, TSS, DS) in both genes (wisp and aq1) for three different antibodies (H3K4me3, H3K9me3 and IgG). There were no significant differences between two groups (HT and LT) for any gene and for any histone modification. P-values of all experimental conditions were over 0.05. The only significant difference was found in wisp1 promoter with IgG antibody, where it is not supposed to be differences. Nevertheless, the trend in all cases was that H3K4me3 presence is higher in HT group than H3K9me3. Furthermore, the results could lead us to think that there are more histone modifications in the TSS region. P-values of statistical tests performed are shown in Annex 3.

Figure 7. ChIP-qPCR analysis of three different regions (PR, TSS, DS) of the two selected genes (A, wisp and B, aq1) from testis of sea bass grown at high and low temperature. Immunoprecipitation was done for 3 different proteins: two histone modifications (H3K4me3 and H3K9me3) and an unspecific antibody (IgG). In the graph the ddCt values with LT group as the reference group are represented, as well as the lower and upper confidence interval.
6 Discussion

Data reported on histone methylation suggested that H3K4me3 is able to activate gene expression, while H3K9me3 is related to gene expression silencing (Rose & Klose, 2014). Our initial hypotheses are summarized in Table 1. We expected in group HT higher levels of H3K9me3 in some regulatory regions of the gene wisp1, which is less expressed in the group HT. In contrast, in the group LT we expected higher levels of H3K4me. With regard to aq1 gene, we expected the same level of each histone modification or a compensation of both. Contrary to expectations, this study did not find a significant difference between group HT and LT. It is needed to take into account that there are many histone modifications which have not been studied. Not only trimethylation of histone H3 has an important effect in gene expression regulation, but also other modifications like acetylation of other histones take part of this regulatory process. Perhaps, studying a wider range of histone modification would lead us to a different result. Furthermore, we have not analysed the whole gene, but three candidate regions of interest. As a consequence, the modifications studied could be in other parts of the gene or at distal regulatory elements known to influence transcription.

On the other hand, all the genes are not regulated by histone modification, there are also other types of epigenetic modification that may be affecting gene transcription. This leads us to think that the expression of each gene or genomic region is different because it is affected by various factors like DNA methylation, other histone modifications, transcription factors, post-transcriptional or post-translational modifications.

<table>
<thead>
<tr>
<th>Hypothetical situation</th>
<th>Expression</th>
<th>H3K4me3 level</th>
<th>H3K9me3 level</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT</td>
<td>wisp1</td>
<td>Lower</td>
<td>Lower</td>
</tr>
<tr>
<td></td>
<td>aq1</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>LT</td>
<td>wisp1</td>
<td>Higher</td>
<td>Higher</td>
</tr>
<tr>
<td></td>
<td>aq1</td>
<td>=</td>
<td>=</td>
</tr>
</tbody>
</table>

*Table 1. Hypothetical situation of the experiment.*

The standard error in the same group is too high due to two different sources of variation: the sample size (n) and the tissue studied. We have only analysed three fishes of each group (n=3). As a consequence, due to a small difference between individuals of
the same group variability increases. Furthermore, we have used testis for the ChIP, which is a tissue with a high cellular heterogeneity. It is likely that using liver or muscle could give more reliable results. At first, we tried to perform ChIP with muscle for our experiment, but we had complications regarding to homogenization step and the small tissue size we had. Since muscle contains less nuclei per volume and all tissue samples available were relatively small, we decided to use testis. However, the tissue size was also small and the experiment was not designed for that. Other studies carried out by Simonet et al. (2013) with liver tissue show less variation in the same group, but also were performed with large fresh tissues (~5ml). It is also necessary to take into account that we have a heterogeneous cell population for the assay with different epigenetic states.

Performance of the ChIP technique has also an important implication in the experiment results. First of all, the results depend on the specificity of the antibodies. Furthermore, there are two ChIP variants. We have carried out N-ChIP, in which the chromatin is sheared by an enzymatic digestion, but it is not fixed with formaldehyde because it can restrict the access of the enzyme to the chromatin (Haring, M. et al., 2007). However, there is another variant called X-ChIP, which is more used in transcription factors studies. In this variant, chromatin is fixed using formaldehyde and it is sheared by sonication. Thus, different chromatin preparation methods can lead to different results. We have chosen N-ChIP after doing several optimizations. ChIP is a procedure that needs a huge amount of DNA, so it is not design for small amount of sample. Therefore, we had to optimized the protocol in order to perform ChIP with testis tissue of the European sea bass. Although the further cleaning help to clean the nuclei sample, it caused the loss of chromatin. Thus, we removed this step in order to achieve the greatest amount of DNA. We have concluded that N-ChIP is more adequate for histone modifications studies. Enzymatic digestion with Micrococcal nuclease is more efficient and less destructive than sonication. Furthermore, formaldehyde cross-linking could have negative effects on immunoprecipitation due to modification of epitopes that have to be recognized by specific antibodies. In N-ChIP it does not happen because fixation is eluded. Moreover, in contrast to Mnase digestion, sonication shearing results in fragments of different size and it may not produce chromatin fragments small enough for the regions of interest (Umlauf, D. et al., 2005). For the chromatin shearing it is necessary to take into account that exceeding the time of sonication or Mnase digestion
could lead us to excessive fragmentation. In our case, 1 Units of Mnase for each microgram of chromatin for 20 minutes were enough. Nevertheless, shearing conditions must be optimized for each case.

We have studied the presence of two histone modification in two genes, whose functions are different. On one hand, *Wnt1 inducible signalling pathway protein 1* (*wisp1*) participates in a pathway whose activation is involved in humans and mouse spermatogonia proliferation and renovation (Sambroni et al., 2013). Furthermore, it has been shown that gonadotropic Follicle-stimulating hormone (FSH) which takes part in spermatogenesis, stimulates *wisp1* gene expression. *wisp1* transcript is expressed in Sertoli cells, where the FHS receptor is found (Sambroni, Lareyre and Le Gac, 2013). It is also known that the Wnt pathway is essential for embryonic development and neurogenesis. Therefore, *wisp1* is a gene involved in development, sexual determination and gonads differentiation. In group exposed at high temperatures, in which there are more females, *wisp1* is less expressed. On the other hand, *Aquaporin 1* (*aq1*) encodes for an integral membrane protein which functions as a molecular water channel. *aq1* is a well conserved gene present from bacteria to mammals. In marine teleosts it is expressed in ovaries and testis, but its regulation and function are different. In gametes and early embryos, in which in contrast to adults lack of osmoregulatory system, aquaporin 1 is essential to allow water influx into the oocyte to compensate for the passive water efflux due to the hyperosmotic situation. *Aquaporin 1* provides a water reservoir in the embryo during the last stages of oogenesis. Furthermore, it has been seen that egg hydration improves oxygen exchange, buoyancy and dispersion (Tingaud-Sequeira et al., 2008). Different aquaporins are abundant in the testis of marine teleost. *aq1* is only expressed in developing germ cells, but is the first expressed in proliferating spermatogonia in seabream. During spermatogenesis, water transport is essential to regulate homeostasis as occurs in oogenesis. These channel proteins are implicated in fluid efflux during the sequential cell divisions from spermatogonia to spermatozoa and in the transport of nutrient from Sertoli cells (Boj et al., 2015). *aq1* is also involved in activating or maintaining the flagella movement (Chauvigné et al., 2013). The early expression of *aq1ab* during seabream spermatogenesis is very similar to the developmental control of the same gene during oogenesis. Therefore, *aquaporin 1* plays an important role in both gonads, testis and ovaries. However, the endocrine control of the two paralog genes is different in each case. During oogenesis the expression of
$aq1ab$ in seabream is upregulated by Follicle-stimulating hormone (FSH), whereas the transcription of $aq1ab$ during spermatogenesis not only could be under Luteinizing hormone (LH) regulation through steroid-independent mechanisms, but also under androgens regulation (Boj et al., 2015).

In conclusion, in this study we did our best using a targeted candidate gene and histone modifications approach to identify potential targets of the temperature response. Targeting specific histone modifications and gene regions was a necessity due to limited sample resources and time, but we failed to detect differences in the 2 histone modifications we assayed, although we detected significant differences in gene expression. Other epigenetic mechanisms, other histone modifications and other genes regions overlooked here may play a role in regulating the expression of $wisp1$ and $aq1$, our 2 genes of interest.
7 Conclusion

Despite the fact that ChIP is an extended used method to study histone modifications, it is mostly used in cell lines and in some cases in tissues, in which there are a great amount of chromatin. The main step of ChIP is purification of nuclei and it could be tricky depending on the tissue structure. After all these experiments we now know that a lot of chromatin or tissue is necessary to perform ChIP and obtain successful results.

The optimization of purification nuclei, chromatin shearing and immunoprecipitation steps described in the ChIP protocol developed for lower amounts of DNA in European sea bass testis has been carried out in this work. According to this protocol, ChIP will be able to be used on studies of histones modifications with limited sample resources. Nevertheless, we suggest to optimize ChIP parameters such as chromatin shearing for each particular case.

The induced temperature regimes (HT and LT) failed to produce histone modifications on the two genes studied (wisp and aqI), at least on the three regions analysed. However, HT animals showed higher affectation on H3K4me3 presence than LT for the three regions studied.

Although other studies have described some effects of temperature in epigenetic cell status we cannot discard that other epigenetic mechanisms, other histone modifications and other genes regions overlooked here may play a role in regulating the expression of wisp1, our gene of interest.
8 Annexes

8.1 Annex 1. Chromatin Immunoprecipitation Protocol

1) Nuclei purification

*Keep the samples on ice during all the procedure.*

- Put the tissue in ice-cold H buffer. The volume depends on the size of the tissue. We can put 250 µl in an 1.5 mL Eppendorf.
- Homogenize tissue using the pistil.
- Centrifuge at 1000 x g for 10 min at 4ºC.
- Discard the supernatant.
- Resuspend in 500 µl of Buffer H

Check de DNA concentration with phenol-chloroform-isoamyl alcohol (PCI) DNA extraction:

- Take 20 µl nuclei sample and add 80 µl of buffer H.
- Add 2 µl Proteinase K (10 mg/ml) and vortex.
- Incubate at 55 ºC O/N.
- Add 1µl of RNAse A (20mg/ml).
- Incubate for 30 min at 55 ºC.
- Vortex digestion mixture and centrifuge at 12000 rpm (14000g) for 1 min.
- Transfer de supernatant (100 µl) to a new microfuge tube and add 100 µl of PCI (25:24:1) and vortex.
- Centrifuge at 12,000 rpm for 5 min.
- While the first tube is spinning, add 100 µl of CI (Chloroform: Isoamyl alcohol at 24:1) to a new microfuge tube.
- Pull off the top layer carefully using a pipette and add to the new tube of CI.
- Mix and centrifuge at 12,000 rpm for 2 min.
- Carefully pull off the top layer of the centrifuged tube and add to a new microfuge tube.
- Add 4 µl of 5M NaCl (fine for 100 µl but you should use proportionally less or more in depending on what you’ve got) + 2,5 volumes of 95% EtOH (e.g. if you had 100 µl of supernatant, add 250 µl of EtOH) and mix thoroughly by inverting (NOT vortexing).
- Incubate at -20ºC for at least 15 min.
- Centrifuge at maximum speed for 5 min (4ºC) and pour off the supernatant of pull off with pipette and discard it.
- Wash with 100 µl of 70% EtOH by carefully pipetting along the side of the tube away from the DNA pellet.
- Centrifuge at maximum speed 3 minutes, pour off supernatant and discard it. Add another aliquot of 100 µl of 70% EtOH.
- Centrifuge the tube again for 2 min and carefully pipet any excess liquid off of the pellet.
- Quick spin the tube and pull off the last few drops of liquid.
- Dry pellet (air dry on the bench, incubate at 37ºC, or use the speed vac on low heat and no vacuum). Ensure there is no residual ethanol present in the tube.
- Resuspend in 20 µl of nuclease free water.
- Quantify the DNA with Nanodrop and check if necessary load the samples in a 1,5 % agarose gel.

2) Mnase digestion
We do the digestion reaction in a volume of 500 µl. The quantity of DNA must be calculated. The digestion conditions have to be optimized. I used 3U/µg chromatin for 15 minutes at 37 ºC.
- Dilute the enzyme Micrococcal nuclease (e.g. 1 µl enzyme in 50 µl of water, so the concentration will be 40 U/µl).
- Separate the sample in two different tubes (250 µl in each one).
- Add 50 µl BSA 100 µg/ml.
- Add 50 µl 10X Micrococcal nuclease Reaction Buffer.
- Calculate the µl of enzyme we need in each reaction in function of the DNA concentration.
- Add water to 500 µl.
- Incubate at 37 ºC for 15 min.
- Add 500 µl Buffer after Mnase digestion 2X.
- Incubate 5 min in ice.
- Centrifuge at 8000g for 5 minutes (4 ºC).
- Recollect the supernatant.

3) Immunoprecipitation (IP)

Day 1

*Equilibration of beads (Dingbats Protein G, 10003D, Life Technologies).*

- Vortex *Dynabeads* for 30 seconds.
- Prepare 1 tube per IP. Add desired amount of beads (30µl beads per IP) to each.
- Add 1ml cold 1X PBS (w/ prot. inhib.). Soft vortex.
- Place the tubes on magnetic stand and invert several times. Allow beads to clump for ~1 min.
- Pipet off PBS. Repeat wash step two more times.
- Add ~ 4µg of antibody per IP. It depends on the antibody quality.
- Adjust to 500 µl with RIPA-150, resuspend *Dynabeads*.
- Incubate on rocker for 6 hours at 4 ºC (prebinding).
- Place the tubes in magnetic rack. Invert several times. Allow beads to clump and discard supernatant.
- Try to use as much chromatin as possible, ideally 20-30 µg, minimum 15 µg, for each point of the IP: Input, IgG and antibody. The input has to be frozen at this point.
- Add chromatin to Ab-bound Dynabeads. Gently mix and place on rocker O/N at 4ºC.

Day 2

- Put tubes on magnetic rack. Invert several times. Allow beads to clump and discard supernatant.
- Perform the following wash steps with 800 µl of cold buffer. Flick tubes to resuspend beads. Incubate each wash for 5 min on rocker at 4 ºC. Place tube in magnetic stand. Invert several times. Allow beads to clump and discard supernatant.
  - 1 time with RIPA-150
  - 2 times with RIPA-500
- Resuspend beads in 200 µl IP Elution Buffer (fresh at RT). Vortex.
- Add 2 µl of RNase A and incubate 4 hours at 65 ºC (briefly vortex sample every 30 minutes for the first 2 hours).
- Quick spin sample. Place in magnetic rack. Allow beads to clump and transfer supernatant to a new tube.
- Add 4 µl Proteinase K and incubate O/N at 55 ºC. Include the INPUT controls.
- Add 2 µl RNase A only to the input and incubate at 55ºC for 30 min.
- Transfer de supernatant (200 µl) to a new microfuge tube and add 200 µl of PCI (25:24:1) and vortex.
- Centrifuge at 12.000 rpm for 5 min.
- While the first tube is spinning, add 200 µl of CI (Chloroform: Isoamyl alcohol at 24:1) to a new microfuge tube.
- Pull off the top layer carefully using a pipette and add to the new tube of CI.
- Mix and centrifuge at 12.000 rpm for 2 min.
- Carefully pull off the top layer of the centrifuged tube and add to a new microfuge tube.
- Add 8 µl of 5M NaCl (fine for 200 µl but you should use proportionally less or more in depending on what you’ve got) + 2,5 volumes of 95% EtOH (e.g. if you had 200 µl of supernatant, add 500 µl of EtOH) and mix thoroughly by inverting (NOT vortexing).
- Incubate at -20ºC for at least 15 min.
- Centrifuge at maximum speed for 5 min (4ºC) and pour off the supernatant of pull off with pipette and discard it.
- Wash with 500 µl of 70% EtOH by carefully pipetting along the side of the tube away from the DNA pellet.
- Centrifuge at maximum speed 3 minutes, pour off supernatant and discard it. Add another aliquot of 500 µl of 70% EtOH.
- Centrifuge the tube again for 2 min and carefully pipet any excess liquid off of the pellet.
- Quick spin the tube and pull off the last few drops of liquid.
- Dry pellet (incubate at 37°C). Ensure there is no residual ethanol present in the tube.
- Resuspend in 40µl of TE buffer.

6) qPCRs

- 2 µl IP DNA
- 2 µl Eva Green
- 0.5 µl Primer F (10 µM)
- 0.5 µl Primer R (10 µM)
- 5 µl H2O MiliQ

We need to dilute input. It should be at 1-10 %.

Buffers

Sucrose solutions
Filter sucrose using a syringe and a 0.45µm filter (0.22µm filter would also serve, but filtering the 2M sucrose would take forever). Change the filter every 10ml of buffer passing through the syringe.

Homogenization Buffer (H Buffer) 4°C
0,25 M Sucrose
0,02 M Tris pH=7,4
3 mM CaCl2
1 Tablet of Complete Protease Inhibitor Cocktail (Roche, USA) for each 50 ml of H Buffer.

Buffer after Mnase digestion 2X
90 mM Hepes pH = 7.9
220 mM NaCl
10 mM EDTA (necessary to stop the enzyme reaction, must be at a final concentration of 5 mM)
1 % Nonidet P-40
0.2 % sodium deoxycholate
0.2 % SDS
**RIPA-150 (4 °C)**
50 mM Tris-HCl pH = 8  
0.15 M NaCl pH = 8  
1 mM EDTA pH = 8  
0.1 % SDS  
1 % Triton X-100  
0.1 % sodium deoxycholate

**RIPA-500 (4 °C)**
50 mM Tris-HCl pH = 8  
0.5 M NaCl pH = 8  
1 mM EDTA pH = 8  
0.1 % SDS  
1 % Triton X-100  
0.1 % sodium deoxycholate

**RIPA-LiCl (4 °C)**
50 mM Tris-HCl pH = 8  
1 mM EDTA pH = 8  
1 % Nonidet P-40  
0.7 % sodium deoxycholate  
0.5 M LiCl

**1X TE Buffer pH = 8 (4 °C)**
10 mM Tris HCl pH = 8  
1 mM EDTA pH = 8

**IP Elution Buffer**
10 mM Tris-HCl pH = 8  
0.3 M NaCl pH = 8  
5 mM EDTA pH = 8  
0.5 % SDS
8.2 Appendix 2

<table>
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<th>Shapiro-Wilk test</th>
<th>t student test</th>
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*Table 2.* P-values of Levene’s, Shapiro-Wilk, t-student and Kruskal Wallis tests done for RT-qPCR data. Values with * are significant.

8.3 Appendix 3

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*Table 3.* P-values of Levene’s, Shapiro-Wilk, t-student and Kruskal Wallis tests done for the ChIP-qPCR data. Values with * are significant.
9 Bibliography

A beginner’s guide to ChIP. Abcam.


