Temperature-independent genome-wide DNA methylation profile in turbot post-embryonic development

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Abstract

The morphological and biological characteristics of ectothermic vertebrates are known to be strongly influenced by environmental conditions, particularly temperature. Epigenetic mechanisms such as DNA methylation have been reported to contribute to the phenotypic plasticity observed in vertebrates in response to environmental changes. Additionally, DNA methylation is a dynamic process that occurs throughout vertebrate ontogeny and it has been associated with the activation and silencing of gene expression during post-embryonic development and metamorphosis. In this study, we investigated genome-wide DNA methylation profiles during turbot metamorphosis, as well as the epigenetic effects of temperature on turbot post-embryonic development. Fish growth and rates of development were greatly affected by rearing temperature. Thus, turbot raised at ambient temperature (18°C) achieved greater body weights and progressed through development more quickly than those reared at a colder temperature (14°C). Genome-wide DNA methylation dynamics analyzed via a methylation-sensitive amplified polymorphism (MSAP) technique were not significantly different between animals reared within the two different thermal environments. Furthermore, comparisons between phenotypically similar fish revealed that genome-wide DNA methylation profiles do not necessarily correlate with specific developmental stages in turbot.

Keywords: turbot, DNA methylation, temperature, metamorphosis, MSAP analysis

1. Introduction

Environmental variation generally produces certain anatomical and behavioral changes in living organisms (Denver and Middlemis-Maher, 2010; Schlichting and Smith, 2002). In the past, these phenotypic responses were commonly attributed to genetic mechanisms, and direct environmental effects that were underlying causes were ignored (Fusco and Minelli, 2010). However, studies over the last several decades have demonstrated that not only genetic, but also epigenetic events might be involved in the such environmental adaptations (Faulk and Dolinoy, 2011). Epigenetic effects are defined as any mechanisms that can modify gene expression without changing the DNA sequence itself and are heritable to subsequent generations (Feil, 2008). Epigenetic mechanisms mainly include DNA methylation, histone modification and the production of non-coding RNAs, which together define chromatin structure and its transcriptional state (Atlasi and Stunnenberg, 2017; Delcuve et al., 2009). DNA methylation is one of the most relevant epigenetic events in vertebrates (Varriale, 2014). High levels of DNA methylation are associated with gene repression, meanwhile reduced levels of methylation are related to gene activation (Razin and Cedar, 1991). This mechanism consists of adding or removing methyl groups (via DNA methyltransferases or demethylases, respectively) to cytosines of CG dinucleotides throughout the genome (Newell-Price et al., 2000).

In fish, recent studies have shown that environmental factor-induced DNA methylation contributes to phenotypic plasticity, causing a variety of morphological and physiological changes. Thus, it has been shown that smoltification in brown trout (*Salmo trutta*) appears to be mediated by global methylation changes under enriched-salt feeding, suggesting that epigenetics underlies anadromy (Morán et al., 2013). Also, phenotypic traits related to sea migration were encoded by differentially methylated loci between smolt and resident rainbow trout (*Oncorhynchus mykiss*) (Baerwald et al., 2016). Another important environmental factor that has been shown to affect the degree of DNA methylation in fish is water temperature.

Water temperature is considered a critical environmental factor affecting fish biology. Fish are ectothermic vertebrates, which means that they cannot maintain a constant body temperature like endotherms. Therefore, fish body temperatures are reflections of the environmental water temperature in which they live (Tattersall et al., 2012). Interestingly, a negative correlation between body temperature and the degree of methylation was shown when polar and tropical fish were compared (Varriale and

Bernardi, 2006). Moreover, environmental water temperature variation during larval development was reported to modify DNA methylation patterns affecting sex determination in Nile tilapia (*Oreochromis niloticus*) (Sun et al., 2016), half-smooth tongue sole (*Cynoglossus semilaevis*) (Shao et al., 2014) and sea bass (*Dicentrarchus labrax*) (Navarro-Martín et al., 2011; Piferrer, 2013), and the expression of genes involved in development and morphogenesis (Anastasiadi et al., 2017). Embryonic temperature-dependent demethylation of the *myogenin* promoter was shown to contribute to muscle development and growth in Atlantic salmon (*Salmo salar*) (Burgerhout et al., 2017).

DNA methylation acts not only as an epigenetic mechanism to control gene expression in response to changing environmental conditions, but it also plays an important role in development and morphogenesis (Cedar and Bergman, 2012). Therefore, epigenetic changes occur throughout important developmental events characterized by specific phenotypic shifts. The early maturation transitory stage in Atlantic salmon was reported to be mediated by DNA methylation (Moran and Perez-Figueroa, 2011). Also, tissue-specific methylation changes were associated with the postembryonic remodeling that some fish, including lamprey (*Petromyzon marinus*) (Covelo-Soto et al., 2015) and European eel (*Anguilla anguilla*) (Trautner et al., 2017), experience during the transition from larval to juvenile stages. These changes require profound transcriptional reprogramming events for fish to acquire new morphological, physiological and behavioral features (McMenamin and Parichy, 2013).

Turbot (*Scophthalmus maximus*) is an economically valuable aquaculture species which undergoes a dramatic metamorphosis throughout the transition from a pelagic and bilateral symmetric larva to a benthonic, asymmetric juvenile (Al-Maghazachi and Gibson, 1984). Whether an epigenetics mechanism, specifically DNA methylation, contributes to turbot larval development is currently unknown. Understanding how epigenetics is associated with turbot development, as well as its susceptibility to be affected by environmental changes may facilitate the improvement of productive aquaculture practices. Therefore, the present study aims to study global DNA methylation patterns during turbot post-embryonic development and elucidate whether they are affected by environmental temperature (*i.e.*, rearing temperature). For this purpose, we performed methylation-sensitive amplified polymorphism (MSAP) analyses of specific larval and juvenile stages in turbot reared at 18°C and 14°C.

2. Materials and methods

2.1 Experimental design

In order to produce high-quality embryos and avoid the possibility of strain- or family-associated differences, single pair mating was performed. Therefore, high quality eggs from a single female were fertilized with high-quality sperm from a single male. Briefly, gametes were obtained in April–May 2010 from turbot broodstock reared in facilities of the Centro Oceanográfico de Vigo (NW Spain). Eggs from one ovulated female and milt from one male were obtained by abdominal massage. Egg quality was assessed according to the criteria of McEvoy (1984). First, 10 mL eggs were coated with 200 μ L milt. Then, each volume of eggs and milt was mixed with 2 volumes of seawater. The timepoint in which seawater was added was considered time zero. Thirty seconds after fertilization, eggs were gently rinsed with sea water. Viability was assessed by placing eggs in a plastic graduated cylinder, letting them incubate for 2 h and measuring the proportion that floated. Subsequently, fertilized eggs were placed in 150 L cylinder-conic incubators (4,000 eggs L⁻¹) where the embryos were allowed to float freely under a constant stream of water maintained at 14°C. Hatching took place over a 5 day-post-fertilization (dpf) period, and this timepoint was considered time zero post-hatching. One day post-hatching (dph), larvae were randomly separated into 4 groups and each group was placed into one of four 450 L tanks (30 larvae L⁻¹) with different water temperatures (two tanks at 14°C and two tanks at 18°C). The experimental temperatures (14°C, cold “C” and 18°C, ambient “A”) were chosen in order to simulate the range of temperatures that turbot encounter throughout development in the natural environment. Further, the temperatures have previously been demonstrated to be adequate for the successful rearing and feeding of fish within aquaculture facilities (Rodríguez Villanueva and Fernández Souto 2019). The regulation of temperature was achieved with the use of a heater-coolant device (Pasquarium®, Vigo, Spain). The fish were fed *ad libitum* initially with rotifers (1–7 dph), rotifers plus newly hatched *Artemia* sp. nauplii (7–10 dph), newly hatched *Artemia* sp. nauplii (10–17 dph), Multigain enriched-artemia (17–30 dph), and from 30 dpf onward with GEMMA-Micron® (Skreting, Norway). Ethical approval (AGL2008-00392/ACU) for all studies was obtained from the Institutional Animal Care and Use Committee of the IIM-CSIC Institute in accordance with the National Advisory Committee for Laboratory Animal Research Guidelines

licensed by the Spanish Authority (RD53/2013) and the work is in conformance with the European animal directive (2010/63/UE) for the protection of experimental animals.

2.2 Fish growth

As a measurement of developmental progress, the wet body mass and developmental stage (Al-Maghazachi and Gibson, 1984) of turbot were determined at 4, 15, 30, 40 and 50 dph using 10 larvae randomly sampled in each temperature group. Developmental progress of turbot samples at different temperatures for each dph timepoint were compared.

2.3 DNA isolation

Individual fish samples at 15 dph (A= 4 and C= 4), 30 dph (A= 10 and C= 10) and 50 dph (A= 8 and C= 10) were euthanized using MS-222 (Sigma) and preserved in absolute ethanol at 4°C. The 15, 30 and 50 dph timepoints selected correspond to pre-metamorphic, metamorphic climax and post-metamorphic stages, respectively, for turbot cultured at 18°C. Whole fish larvae were sliced and homogenized. Genomic DNA was extracted from resulting homogenized material using the QIAamp DNA kit (Qiagen). DNA quality was verified by electrophoresis on 1% agarose gels. Subsequently, DNA concentration was quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific) and sample concentrations were adjusted to 50 ng μL^{-1} .

2.4 Methylation-sensitive amplified polymorphism (MSAP) analysis

Genome-wide analysis of DNA methylation was carried out according to the MSAP protocol described by (Moran and Perez-Figueroa, 2011), which is a modified version of previously described methodologies (Reyna-López et al., 1997; Xu et al., 2000). The MSAP technique is based on the use of two cytosine methylation-sensitive restriction endonucleases, HpaII and MspI, to detect DNA methylation fingerprints. HpaII and MspI are isoschizomers which recognize the same sequence (5'-CCGG sites) but display differential sensitivity to DNA methylation. HpaII is sensitive to internal cytosine methylation, whereas MspI is sensitive to external cytosine residues that are methylated in hemimethylated targets (*i.e.*, sites methylated only at one DNA strand) (<http://rebase.neb.com>) (Roberts et al., 2015). Also, HpaII and MspI are sensitive to hypermethylated sites (*i.e.*, both at the internal and external cytosines), whereas

unmethylated targets are digested by both enzymes. Therefore, the restriction profiles for each single enzyme allow for an assessment of the methylation state of the target fragments.

For each sample, 50 ng of DNA was independently digested with both EcoRI/MspI and EcoRI/HpaII enzyme combinations (New England Biolabs) and ligated to specific adaptors in parallel reactions as described in Morán et al. (2013). Afterward, restriction fragments were amplified through two consecutive PCR reactions. The first included a pre-selective amplification and the second involved a selective PCR amplification (Morán et al., 2013). Primer sequences used for pre-selective and selective PCR reactions are available in Moran and Perez-Figueroa (2011). Finally, 2 µl of the labelled MSAP PCR product was combined with 15 µl of HiDi formamide and 0.5 µl of GeneScan 500 ROX size standard (Applied Biosystems). Samples were denatured at 95°C for 5 min and cooled on ice. Samples were fractionated on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) at 15 kV for 6 s and at 15 kV for 33 min at 66°C. MSAP restriction profiles were scored using GeneMapper v.3.7 software (Applied Biosystems). DNA fragments less than 100 bp in length, longer than 500 bp or less than 70 RFU (relative fluorescent units) were excluded from the analysis to assure technical reproducibility.

2.5 Quantitative real-time PCR

Turbot of each chronological age and temperature group (n = 10) were fixed in Trizol Reagent (Ambion) and preserved at -80°C for quantitative real time PCR (qRT-PCR) analysis. Total RNA was extracted, and first-strand cDNA was synthesized using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's protocol using 0.5 µg of total RNA. cDNA samples were purified using QIAquick PCR purification columns (Qiagen) following the manufacturer's guidelines. Samples were amplified in triplicate. Briefly, 12.5 µl of Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific) was combined with 0.5 µl 0.2 µM of each primer, 10.5 µl nuclease free water and 1 µl of cDNA template. qRT-PCR reactions were analyzed using a 7500 Fast Real-Time PCR System (Applied Biosystems) with the following cycling conditions: initial denaturation at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Expression levels of the *dnmt1* gene were assessed in two independent experiments. Relative mRNA expression

levels normalized to the housekeeping *18S* ribosomal gene were analyzed using the efficiency calibrated method as previously described (Pfaffl, 2001). Primer sets used for each gene are listed in Table 1.

2.6 Data analyses and statistics

MSAP restriction profiles were assessed using a band-based strategy (Bonin et al., 2007) for products generated using EcoRI-HpaII and EcoRI-MspI primers independently. The resulting presence/absence matrices were integrated and analyzed with the R software package, *msap v.1.1.4* (Perez-Figueroa, 2013), using its scoring procedure. Every band was scored as follows: present in both EcoRI-HpaII and EcoRI-MspI products (1/1), denoting non-methylated state (NMT); present only in either EcoRI-HpaII (1/0) or EcoRI-MspI (0/1) products, corresponding to hemimethylated (HMM) or internal cytosine methylation state (ICM). The absence of both bands (0/0) was considered as hypermethylated state (HPM). Loci were classified as methylation-sensitive loci (MSL) versus the non-methylation-sensitive loci according to whether the observed proportion of methylated states was greater than 5% (error rate-based threshold). Only MSL showing at least two occurrences of each state (*i.e.*, polymorphic loci) were selected for further analysis.

To study the DNA methylation pattern differences among experimental groups (the combination between experimental temperatures and days post-hatching), pairwise analyses of molecular variance (AMOVA) and principal coordinates analyses (PCoA) were performed. Furthermore, locus-specific methylation differences between experimental groups were assessed using the Fisher's exact test. After the false discovery rate (FDR) correction method (Benjamini and Hochberg, 1995) was considered, only MSL with a *p*-value < 0.01 were selected, given the number of samples for robust *p*-values. Estimates of relationships among selected loci from the four categories (NMT, HMM, ICM or HPM) were computed using Gower's coefficient of similarity. The resulting distance matrix was used to perform hierarchical clustering using an unweighted pair group method with arithmetic mean (UPGMA) and was visualized as a heatmap matrix with the R "ComplexHeatmap" package (Gu et al., 2016). A summary of all of the code needed to reproduce the analyses described above is given in the Supplementary Report.

To analyze growth using weight measurements and quantitative gene expression analyses, data were expressed as mean $\pm$ standard error of the mean (SEM). Multiple

comparisons between numerical data were evaluated using one-way ANOVA with the Tukey's post hoc test. Data are statistically similar if they share at least one letter. A $p \leq 0.05$ (letters) is considered to be statistically significant. Statistical analyses and figures were performed with R Studio (R statistical software v3.3.2) and SigmaPlot 12.0.

3. Results and Discussion

3.1 Thermal environment influences turbot post-embryonic development

Fish are ectotherms and water temperature variations generally affect their growth and development (Boltaña et al., 2017; Kamler, 2002). Here, fertilized eggs of turbot were exposed at 1 dph to two distinct rearing temperatures (14°C or 18°C) and, subsequently, the survival, growth and development of larvae were assessed throughout post-embryonic stages. Mortality was monitored daily and no significant differences were observed between experimental groups (percentage mortality = $35 \pm 3\%$). The critical period for survival was at early stages of larval development (2–8 dph), throughout which the highest levels of mortality were recorded. Fish were sampled, weighed and staged at 4, 15, 30, 40 and 50 dph. The wet body mass of turbot larvae reared at 14 and 18°C is shown as a function of chronological age (Fig. 1A). No significant differences in body mass were found until 15 dph between the two temperature groups (Fig. 1A).

After 15 dph, significant differences in mean wet body mass were recorded between the two temperature groups. Thus, fish of same chronological age reached higher body weight when grown at water temperature four degrees higher. As thermal environment largely affects not only the growth (Boltaña et al., 2017), but also the developmental rate in fish (Kamler, 2002; Seikai et al., 1986), we specifically analyzed the developmental progress of each of the two groups. We found that fish of same chronological age were at a more advanced developmental stage when cultured at 18°C compared with 14°C (Fig. 1B). The most remarkable difference was found at 30 dph. While 30 dph larvae raised at ambient temperature (18°C) were almost fully metamorphosed juveniles (S4–4d), at cold temperature (14°C) 30 dph larvae were still at the beginning of the metamorphic phase (S4–4a) (Fig. 1B). Moreover, a comparison between larvae displaying a similar morphology and growth under the different experimental temperatures revealed that 15 and 30 dph turbot reared at 18°C were at virtually the same developmental stage (gray boxes in Fig. 1B) and were similar body weights (gray boxes in Fig. 1A) as those grown at 14°C for 30 and 50 d, respectively.

Our results show that the phenotypic plasticity of turbot development is affected by temperature, as previously reported in this and other teleost fish species (Pittman et al., 2013). Similarly, increased rearing temperature (from 14°C to 22°C) modified turbot growth, developmental rate and the timing of metamorphosis (Ayala et al., 2016; Torres-Núñez et al., 2014) as well as affecting the number of dorsal and anal fin rays produced (Torres-Núñez et al., 2014) and muscle cellularity (Ayala et al., 2016). Developmental progress throughout larval stages and overall growth were enhanced by post-hatching temperature increases in other flatfish species including Japanese flounder (*Paralichthys olivaceus*) (Seikai et al., 1986) and northern rock sole (*Lepidopsetta polyxystra*) (Laurel et al., 2014). Exposure to high temperatures stimulated muscle growth in *Dicentrarchus labrax* larvae (Alami-Durante et al., 2006). In *Salmo salar*, a wide thermal range increased fish size, muscle structure and growth in post-hatch larvae and juveniles (Boltaña et al., 2017). Somatic growth plasticity during embryonic and early larval stages often has important consequences for later size-dependent ontogenetic events. In the present study, we found that different thermal environments modified the onset of metamorphosis, settlement and larval stage duration in turbot. Epigenetic mechanisms are usually behind the phenotypic shifts (Gavery and Roberts, 2017), however, it remains unknown whether the temperature-induced changes in turbot post-embryonic developmental progress are associated with dynamic changes in genome-wide methylation patterns.

3.2 Temperature does not affect genome-wide DNA methylation dynamics during turbot post-embryonic developmental progress

Next, we wondered if rearing temperature was responsible for methylation-mediated ontogenetic changes in turbot post-embryonic development. Although the thermal environment has previously been reported to modify developmental progress and growth in fish (Boltaña et al., 2017; Kamler, 2002), the interaction among temperature, genome-wide DNA methylation dynamics and developmental progress has not been widely studied. To this end, we analyzed genome-wide DNA methylation patterns using MSAP genotyping during development in turbot exposed to ambient (18°C) and cold (14°C) temperatures from hatching to the completion of metamorphosis. The MSAP analyses were performed using two primer combinations and revealed a total of 701 loci. Of these loci, 686 were classified as methyl sensitive loci (MSL) and the remaining 15 were classified as non-methyl sensitive loci (NML). The proportion of polymorphic

MSL used for the analysis was 49% (335 loci). The analysis of different methylation states (*i.e.*, non-methylated, hemimethylated, internal cytosine methylation and hypermethylated states) at the target loci revealed a similar frequency of methylated and unmethylated states within the two experimental groups (18°C ambient and 14°C cold temperatures) at 15, 30 and 50 dph (Table 2). An increase of hypermethylated loci at 15 dph and 50 dph, as well as a larger proportion of methylated internal cytosine residues and hemimethylated states at 30 dph were found in turbot from both experimental temperatures (Table 2). Specifically, multiple pairwise comparisons among all experimental groups showed significant differences in genome-wide DNA methylation dynamics within ambient temperature groups (A-15, A-30 and A-50), and those from cold temperature (C-15, C-30 and C-50) (Table 3). Moreover, the most significant changes in genome-wide DNA methylation patterns were found within experimental groups C-50 and A-30 ($\Phi_{ST} = 0.4066$, $p < 0.0001$), as well as C-30 and C-50 ($\Phi_{ST} = 0.2733$, $p < 0.0001$) (Table 3). According to multiple pairwise comparisons, PCoA analysis (Figure S1 in the Supplementary Report) revealed no epigenetic variation between experimental groups grown in the two temperatures tested, but did show differences among chronological age groups within specific temperature treatment groups (as shown by a hierarchical AMOVA, Table S1 in the Supplementary Report). Further, a total of 220 statistically significant MSAP loci ($p < 0.01$ after applying an FDR correction) were identified using the Fisher's exact test. These 220 differentially methylated loci were clustered within the heatmap in two major groups (15 and 50 dph vs. 30 dph) with all the specimens but one clustering together according to developmental timing (Fig. 2). Within those clusters there did not seem to be a clear division with regard to temperature, confirming the results from the AMOVA and PcoA. In the heatmap, MSAP loci clustered according to methylation state. It is not easy to elucidate global patterning, but there were some clear trends observed. For example, the rightmost cluster consisting of 45 loci within the heatmap displayed a high proportion of hypermethylated states (blue) at 15 dph, which transitioned to mostly non-methylated (orange) states at 30 dph. Further, half of the sites reverted to hypermethylated (blue) states by 50 dph.

Taken together, results from the MSAP analysis show that genome-wide DNA methylation patterns are rearing temperature-independent and vary throughout the process of turbot development. Further, methylation status is more similar at 15 and 50 dph (mostly hypermethylated loci) than 30 dph (internal cytosine methylated and

hemimethylated loci increase). While it is true that 4°C rearing temperature variation did not cause changes in the MSAP methylation patterns across post-embryonic development in turbot, further work assessing gene-specific methylation could unveil possible correlations between rearing temperature and DNA methylation. Moreover, the obvious growth delay observed in turbot larvae reared at cold temperature compared with ambient temperature does not seem to be produced by genome-wide, differential DNA methylation. A reasonable explanation for the observed growth delays could be that water temperature drops directly promote biochemical changes, and therefore, slow the developmental progress. The fact that environmental temperature controls biochemical reactions and metabolic rate in ectotherms has been shown, thus, fish growth and other physiological processes could be the result of adaptation by metabolic machinery to a new thermal environment (Angilletta, 2009; Boltaña et al., 2017). Additionally, by assessing the size and chronological age of fish from both experimental groups, one could hypothesize that genome-wide DNA methylation profiles in turbot are probably developmental stage-specific. However, comparisons of larvae of similar developmental stages under different experimental temperatures (A15 vs. C30 and A30 vs. C-50) (gray boxes in Fig. 1B) presented significantly different genome-wide DNA methylation profiles (A15 vs. C30: $p = 0.0013$; A30 vs. C50: $p < 0.0001$) (Table 3). Consequently, these data suggest that genome-wide DNA methylation profiles correlate with the chronological age in turbot. Therefore, they might not be completely associated with the metamorphic remodelling process itself, as has been observed in other fish species (Covelo-Soto et al., 2015; Trautner et al., 2017). Further, the age-specific DNA methylation profile could be maintained through DNA methyltransferase 1 (DNMT1, maintenance methyltransferase) (Newell-Price et al., 2000). Analysis of *dnmt1* transcripts by quantitative RT-PCR showed a similar gene expression pattern in turbot reared at ambient (18°C) and cold (14°C) temperatures (Fig. 3). Specifically, significant differences in the expression of the *dnmt1* gene were found at 30 dph when compared with previous and later stages. At 30 dph, *dnmt1* gene reached its maximum level of expression, which presumably contributed to internal cytosine methylation and hemimethylation states observed.

4. Conclusions

We have studied, for the first time, the genome-wide DNA methylation profile throughout post-embryonic development and the effects of rearing temperature (18°C,

ambient, and 14°C, cold, temperatures) on methylation status in turbot. The results revealed a temperature-independent genome-wide methylation pattern for turbot that occurred during larval and juvenile development. Epigenetic differentiation based on MSAP analysis among experimental groups showed that a 4°C water temperature change did not cause significant degrees of variation of ontogenetic genome-wide DNA methylation patterns. Additionally, rearing temperature strongly affected fish growth and developmental rates. Therefore, taken together, the genome-wide DNA methylation profile is supposed to correlate with the chronological age to a greater degree than developmental stage in turbot.

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Table 1. Primer sequences used for quantitative RT-PCR gene expression analyses

Genes	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
<i>dnmt1</i>	AGGACGTGGATGAGGATTTG	AAGTGGATTTGACGCCTTTG
<i>18S</i>	AAACGGCTACCACATCCAAG	CCTCCAATGGATCCTCGTTA

Table 2. Methylation status of target sequences (percentages) from each experimental group

Band pattern (target state)	A-15	A-30	A-50	C-15	C-30	C-50
HPA+/MSP+ (Non-methylated)	10.2	15.2	13.1	12.0	13.0	13.1
HPA+/MSP- (Hemimethylated)	9.0	13.2	10.9	7.8	12.4	10.1
HPA-/MSP+ (Internal C methylation)	12.1	16.1	10.4	11.5	17.0	11.8
HPA-/MSP- (Hypermethylation)	68.7	55.5	65.6	68.7	57.6	65.0

Table 3. Pairwise AMOVAs between all pairs of experimental groups

	A-15	A-30	A-50	C-15	C-30	C-50
A-15		0.0009	0.001	0.9717	0.0013	0.0009
A-30	0.4704		<0.0001	0.0012	0.0132	<0.0001
A-50	0.3317	0.4668		0.0017	<0.0001	0.0042
C-15	-0.0541	0.46742	0.3620		0.0011	0.0009
C-30	0.3197	0.0805	0.3183	0.3296		<0.0001
C-50	0.2751	0.4066	0.1277	0.3029	0.2733	

Values of Φ_{ST} below the diagonal. *P-values* above the diagonal. Values of $p < 0.01$ have been indicated in bold.

Figure legends

Figure 1. Effect of rearing temperature on body mass and post-embryonic developmental progress in turbot. (A) Body mass of turbot during larval and juvenile development related to days after hatching at (A) ambient temperature (18°C, ●) and (C) cold temperature (14°C, ○). (B) Relationship between days after hatching and post-embryonic developmental stage for turbot reared at 18°C (●) and 14°C (○). Exponential regression analysis revealed a good fit, $R^2 = 0.9996$ (●) and $R^2 = 0.9926$ (○) for days post-hatching (dph) and developmental stages. Rearing temperature group values with different ages (dph) but similar body masses and developmental stages are denoted using grey bars. Fish were staged according to Al-Maghazachi & Gibson (1984).

Figure 2. Heatmap of 220 statistically significant methylation-sensitive loci (MSL) among experimental samples identified using the Fisher's exact test ($p < 0.01$ after FDR correction). Specimens (rows) and loci (columns) were hierarchically clustered using the unweighted pair group method with arithmetic mean (UPGMA). Rearing temperature (A = 18°C and C = 14°C), days post-hatching (15, 30 and 50) and loci methylation status (HMM, hemimethylated; HPM, hypermethylated; ICM, internal cytosine methylation and NMT, non-methylated) are shown in right side of the figure.

Figure 3. Quantitative RT-PCR gene expression analysis of the *dnmt1* gene across turbot post-embryonic development (4, 15, 30 and 50 dph) at ambient (18°C) and cold (14°C) temperatures. Results normalized to *18S* are expressed as the mean $\pm$ SEM of two independent experiments. Data from the 4 dph turbot was set at 1.

A

B

MSAP loci

Specimen

methylation

HMM

HPM

ICM

NMT

temperature

A

C

days

15

30

50