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1 **SPERMATOZOA METHYLOME AND ITS SENSITIVITY TO WATER TEMPERATURE IN A TELEOST FISH**

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6

7 **ABSTRACT**

8 Global climate change and heat waves are sources of stress which fish are facing in the wild as well as  
9 in aquaculture context. In coping with important environmental variations, they demonstrate a great  
10 plasticity and a tendency for acclimation throughout generations. Here, we question whether fish  
11 might be prone to transmit epigenetic alterations through their gametes to their offspring, thus driving  
12 rapid environmental adaptation. The question of epigenetic inheritance in fish has become of crucial  
13 interest in the recent years, when the mammalian model of methylome erasure in germ cells and  
14 embryos was found not to be conserved. In this work, by sequencing spermatozoa after bisulfite  
15 conversion, we characterized the methylation landscape of the paternal gamete in rainbow trout (in  
16 comparison to muscle) before to demonstrate its sensitivity to a 4°C increased rearing temperature  
17 during spermatogenesis. We found that spermatozoa methylome specifically primes housekeeping  
18 and developmental genes for activation and might be instrumental to early development. Most of  
19 these methylation-free promoters were not affected by temperature, attesting the robustness of the  
20 epigenetic programming of early development. However, the increase of temperature triggered the  
21 differential methylation of 5,359 regions, among which 560 gene promoters control spermiogenesis  
22 and lipid metabolism. We therefore report, for the first time in fish, that sperm epigenetic landscape  
23 carries marks of parental thermal living conditions, suggesting that DNA methylation might be a  
24 molecular basis of intergenerational inheritance.

25 **KEY WORDS**

26 Spermatozoa, DNA Methylation, Temperature, Inheritance, Fish

27

28 **HIGHLIGHTS**

29 - Rainbow trout sperm methylome is sensitive to temperature

30 - Developmental gene promoters are not affected by a temperature increase

31 - Spermiogenesis and lipid metabolism gene promoters are modulated by a temperature increase

32 - Sperm methylome might be a molecular vector of transgenerational acclimation to environmental  
33 change

34

35 **INTRODUCTION**

36 Fish demonstrate a great phenotypic plasticity, which allows them to cope with important  
37 environmental variations. At the basis of this plasticity, lies the capacity of the genomes to interact  
38 with the environment through epigenetic mechanisms. Epigenetic landscapes indeed regulate cellular  
39 transcriptional programs and phenotypes. They rely on DNA methylation and histone post-  
40 translational modifications, and are either inherited from a mother cell or modulated upon  
41 physiological context.

42 Increasing evidence indicates that epigenetic information, and *a fortiori* its alteration, are  
43 transmittable through meiosis and fertilization, from generation to generation. This is particularly  
44 suggested by environmental acclimation studies in fish, where offspring performances were shown to  
45 be influenced by the life conditions of their genitors. In the tropical reef fish *Acanthochromis*  
46 *polyacanthus*, the aerobic scope of individuals raised from hatching to adulthood at higher

47 temperatures (+1.5°C and 3°C) is increased if their parents were also reared at these increased  
48 temperatures (Donelson et al., 2012). In the stickleback *Gasterosteus aculeatus*, eggs hatching success  
49 and embryo size are influenced by both maternal and paternal exposure to increased temperature  
50 (Shama and Wegner, 2014). In the tongue sole *Cynoglossus semilaevis*, thermally-induced sex-reversed  
51 pseudo-males have sex-reversed pseudo-male offspring even when these larvae are not exposed to  
52 thermal induction (Chen et al., 2014). In the rainbow trout *Oncorhynchus mykiss*, offspring  
53 thermotolerance (survival and growth) is improved when genitors also have experienced increased  
54 rearing temperature during gametogenesis (Butzge et al., 2021). These data argue in favor of an  
55 intergenerational epigenetic inheritance, which suggests that gametes can transmit environmental  
56 acclimation clues to the offspring. Very few studies investigated fish gametes epigenetics. So far,  
57 Fellous *et al.* reported the differential expression of various epigenetic modifiers in gonads upon  
58 increased temperature in stickleback (Fellous et al., 2022), while Gavery *et al.* found in rainbow trout  
59 differentially methylated DNA regions in spermatozoa from males of wild versus hatchery origins  
60 (Gavery et al., 2018), further indicating that paternal gametes DNA methylome can be modulated by  
61 the environment and might be a molecular vector of intergenerational epigenetic inheritance and  
62 evolution in fish.

63 Primarily often neglected, the importance sperm DNA methylome for offspring development was  
64 recently demonstrated in zebrafish by a series of studies which revealed that, in contrast to  
65 mammalian models, embryo DNA methylome is inherited from spermatozoa, maternal DNA  
66 methylome being remodeled before zygotic genome activation to match the paternal one (Jiang et al.,  
67 2013; Liu et al., 2018; Potok et al., 2013). In addition, zebrafish do not undergo global DNA methylation  
68 reprogramming of primordial germ cells, unraveling the persistence of the epigenetic memory  
69 throughout generations (Ortega-Recalde et al., 2019). For a better understanding of the molecular  
70 basis underlying fish intergenerational inheritance, data reflecting the full diversity of fish species and  
71 reproduction modes are missing, and particularly in the omics perspective.

72 Given the potential importance of the paternal methylome in fish and in the context of environmental  
73 change, we sought to characterize the rainbow trout (*O. mykiss*) sperm methylome and its sensitivity  
74 to a water temperature increase. To this aim, we performed whole genome bisulfite sequencing  
75 (WGBS) on sperm DNA of trout reared at 12°C or 16°C during gametogenesis. Our strategy included  
76 the sequencing of muscle samples from fish raised at 12°C, which was instrumental to identify  
77 spermatozoa-specific DNA methylation profiles. We therefore used these single-base resolution data  
78 sets to (i) characterize genome-wide methylation dynamics in representative rainbow trout germline  
79 and somatic tissues, (ii) highlight the paternal gamete methylome specificities, (iii) assess its sensitivity  
80 to a water temperature increase.

81

## 82 **RESULTS**

### 83 **Rainbow trout genome features and WGBS datasets**

84 We briefly summarize here the main features of publicly available rainbow trout genome, which are  
85 susceptible to impact DNA methylation. *Oncorhynchus mykiss* genome is made of 2.2 Gb parsed into  
86 29 chromosomes with 41,365 and 5,977 coding and non-coding genes, respectively (according to  
87 ensembl v105.1 genome annotation of Omyk\_1.0 version by Full genebuild). It contains 70,672,452  
88 CpGs (70,671,452 nuclear; 1,118 mitochondrial), with a global genome-wide observed over expected  
89 (o/e) CpG ratio of 0.8, indicative of a slight CpG erosion during evolution.

90 We performed WGBS of sperm (12 °C and 16°C) and muscle (12°C) DNA samples according to the  
91 experimental design presented in **Suppl Fig 1**. WGBS yielded a minimum of 300 million paired-end  
92 reads of 150bp per sample (**Tab 1**). In all sample types, the average mapping efficiency of the trimmed  
93 reads was of approximately 60%. Our final data set contains 65,125,263 CpGs, representing 92% of the  
94 trout genome, 70 to 79 % of them being covered at least 5 x. On average, we obtained a sequencing  
95 depth of 10-11 x per strand.

96 We found that almost all methylated cytosines sit in CpG dinucleotides. Indeed, while we measured  
97 average methylation of respectively 87.10 and 74.50 % in CpG context in sperm and muscle,  
98 respectively, methylated CHG and CHH (H representing A, T or C) were detected at rates lower than  
99 1% (**Suppl Tab 1**). For this reason, this study is dedicated to the analysis of CpG methylation only.  
100 Finally, we detected very low CpG methylation on mitochondrial DNA, both in muscle and in  
101 spermatozoa (**Tab 1**). Our sequencing depth analysis revealed an important mitochondrial DNA over-  
102 representation in muscle *versus* sperm (**Tab 1**). This agrees with the expected differences in ratios of  
103 nuclear to mitochondrial DNAs between these tissues with abundant mitochondria in muscle while  
104 trout spermatozoa possess a unique semicircular mitochondrion.

105 **Supplementary Table 2** recapitulates, for each sample, the number of cytosines in CpG context, being  
106 sequenced as methylated in at least one read. When considering the whole data sets, *circa* 93 % of  
107 CpGs, in both tissues, showed at least one positive methylation call. Remarkably, these numbers  
108 decreased as the coverage threshold applied to the data increased (5 x; 10 x), and they dropped faster  
109 in muscle than in spermatozoa. In fact, among the cytosines covered at least 10 times, 67 % of them,  
110 on average, were sequenced as methylated in at least one read in muscle, *versus* 85 % in spermatozoa.  
111 This very reproducible observation among fish samples reveals a fragment selection bias in the  
112 sequencing library preparation process, likely reflecting differences in the chromatin organization  
113 between tissues. Non-methylated DNA fragments are more likely to lie in open and accessible  
114 chromatin, more prone to be resuspended in solution and to be represented in the sequencing  
115 libraries. Both muscle and spermatozoa WGBS libraries show a better representation of non- or poorly-  
116 methylated CpGs, but interestingly, this bias is much milder in spermatozoa, reminiscent of a less  
117 heterogeneous chromatin conformation in this cell type.

118

119 **Rainbow trout genome is globally methylated both in spermatozoa and muscle**

120 To our knowledge, we present the first comprehensive rainbow trout methylomes of somatic and  
121 germline representative cell types. We therefore questioned the general trout DNA methylation  
122 pattern in a descriptive analysis of sperm and muscle of fish reared in control condition (**Suppl Fig 1,**  
123 **fish 1 to 4**). We first assessed the methylation profile on various genomic features, by calculating the  
124 average methylation rates along a reconstituted metagene (**Fig 1A**), around transcription start sites  
125 (TSS) and transcription end sites (TES) (**Fig 1B**) of 115,853 annotated Ensembl coding and non-coding  
126 transcripts (ensembl v104.1, Omyk\_1.0). As already suggested in **Tab 1**, we observed a global DNA  
127 hypermethylation of sperm compared to muscle. This result is consistent with the frequently reported  
128 high methylation level of spermatozoa in vertebrates (Jiang et al., 2013; Kobayashi et al., 2012; Potok  
129 et al., 2013). Nevertheless, both tissues showed a drastic drop of CpG methylation on proximal  
130 promoter regions (1kb upstream of TSS) while gene bodies exhibited particularly high methylation  
131 rates (**Fig 1 A-B-C**). Repeat elements identified using RepeatMasker (Smit, 2015) were all found to be  
132 methylated in both tissues (**Fig 1D**). Transposons, retroposons, LTR, LINEs and SINEs were particularly  
133 highly methylated in sperm. Therefore, our analysis of one somatic and one germline tissue showed  
134 that, like in most Vertebrates, 5mC in rainbow trout mostly occurs in the CpG dinucleotide context,  
135 and shows a global methylation pattern, especially high on gene bodies, and repetitive elements, while  
136 gene promoters are more heterogenous and more likely tend to be methylation-free.

137

### 138 **The signature of sperm methylation profile reflects spermatogenesis and embryonic development**

139 Seeking for spermatozoa methylome specificities we then ran a comparative analysis of sperm and  
140 muscle coupled WGBS data (**Suppl Fig 1B, fish 1 to 4**), from fish reared in control condition. A first  
141 approach, by principal component analysis (PCA), led us to observe a clear segregation of sperm and  
142 muscle samples in spite of a greater dispersion of muscle samples, likely reflecting the greater  
143 complexity of this multicellular tissue compared to sperm (**Fig 2A and Suppl Fig 2**).

144 Differentially methylated CpGs (DMCs) between muscle and spermatozoa were identified by DSS  
145 (Feng et al., 2014; Wu et al., 2015). We found 36,195,141 DMCs with a threshold adjusted p-value of  
146 0.05, meaning that approximately half of the genome is differentially methylated between muscle and  
147 spermatozoa. These DMCs are spread over every chromosome (not shown) and across all genomic  
148 features (**Fig 2B, C**). As expected, most of DMCs (98 %) are hypermethylated in sperm compared to  
149 muscle. Interestingly, while DMCs hypermethylated in spermatozoa are preferentially found in  
150 intergenic regions (also representing the largest part of the genome) (**Fig 2B**), the few sperm-specific  
151 hypomethylated DMCs, are significantly enriched in gene bodies and promoters. This is indicative of a  
152 structural role of sperm DNA hypermethylation on whole genome chromatin. On the contrary, the  
153 enrichment, in spermatozoa, of hypomethylated DMCs in genes and gene-proximal sequences  
154 suggests that a few promoters specifically escape the global methylation and might be instrumental  
155 for the proper execution of spermatogenic transcriptional program. In order to gain insight into this  
156 spermatozoon-specific transcriptional regulation, we isolated promoters and compared their  
157 methylation levels in both tissues. Promoter methylation status is indeed informative of a  
158 transcriptionally repressed or permissive state (however still sensitive to histone post-translational  
159 modifications).

160 Common hyper-methylated promoters (53862 transcripts, **Fig 3A**, orange rectangle, **Suppl Tab 3A**)  
161 were particularly enriched in GO terms referring to cellular transport, cell adhesion and migration,  
162 signaling, response to stimulus, homeostasis, metabolism and terminal differentiation (angiogenesis,  
163 pulmonary valve differentiation, melanocyte differentiation).

164 On the other side, 34,925 common hypo-methylated promoters (**Fig 3A**, blue square, **Suppl Tab 3B**)  
165 were strongly enriched in GO annotations referring to development (morphogenesis, axis  
166 specification, organ development and cell differentiation), germline and housekeeping functions  
167 (transcription, translation, metabolism, DNA repair, cell cycle, proliferation, etc). Some signaling,  
168 response to stimulus and transport terms also appeared enriched in this category. These data reveal



169 that many genes supporting early development or housekeeping functions are by default in a  
170 permissive state for transcription. Even genes which are not necessarily required in the non-  
171 proliferative spermatozoa can nevertheless be primed for transcription in order to support embryo  
172 development. As an example, TSS region of *pola2*, a gene that encodes a subunit of the DNA  
173 polymerase, is equally hypomethylated in both tissues (**Fig 3B**). A vast majority of transcription factors  
174 instrumental for development (*otx*, *cbx* or *pax* families) also have constitutively hypo- or low-  
175 methylated promoters, even though they are not constitutively expressed. Their expression levels  
176 might be regulated by additional post-translational histone modifications as cells differentiate or face  
177 particular contexts.

178 Spermatozoa-specific hypo-methylated promoters belonging to 861 transcripts (**Fig 3A**, green shape,  
179 **Suppl Tab 3C**) were enriched in spermatozoa specific terms such as meiosis and germline, in addition  
180 to development, cellular metabolism and IGF signaling. Some of them are markers of the germline,  
181 such as *piwil1* involved in transposable elements silencing during gametogenesis, *dazl* which encodes  
182 a master translational regulator during spermatogenesis or the sex determining gene *dmrt1* (**Fig 3B**).  
183 Others, such as *spo11* or *mei4* encode specific meiotic recombination factors (**Fig 3B**). We also found  
184 the promoters of the pluripotency factors encoding *klf4* (**Fig 3B**) and *esrrb* to be specifically  
185 hypomethylated. Noticeably, a subset of developmental factors escapes constitutive hypomethylation  
186 and is specifically reprogrammed to be hypomethylated in the spermatozoa. This is the case of the  
187 entire *hox* clusters (**Fig 3B**), in which remarkably, altogether promoters: gene bodies and intergenic  
188 regions, spanning hundreds of kilobases are fully demethylated. The conserved *mir10b*, lying in the  
189 vicinity of *hox4* paralogs and co-expressed during development is therefore hypo-methylated as well.  
190 Interestingly *meis1a* (**Fig 3B**) or *meis3*, which are *hox* cofactors involved in axis patterning and organ  
191 morphogenesis, are also specifically hypomethylated in the spermatozoa. This set of spermatozoa-  
192 specific genes is then silenced by methylation in the adult differentiated muscle, meaning that they  
193 might be sensitive to a dynamic regulation by promoter methylation.

194 Finally, 632 muscle-specific hypo-methylated promoters (**Fig 3A**, red shape, **Suppl Tab 3D**) were  
195 enriched in terms referring to terminal differentiation, muscle and vasculature function, cellular  
196 metabolism, response to stimulus and immune response. These dynamic promoters have undergone  
197 a demethylation during differentiation and are likely to be controlling muscle function. They mainly  
198 regulate terminal differentiation genes, such as *plecb* encoding a skeletal muscle plectin or signaling  
199 molecules such as *rarg* encoding a retinoic acid receptor (**Fig 3B**).

200 Altogether this work strongly supports the idea that sperm methylome is carrying an epigenetic  
201 program, which might be instrumental for both spermatogenesis and proper embryo development.

202

### 203 **Increasing rearing temperature alters sperm methylome**

204 Given the importance of the paternal methylome in fish, we sought to question its robustness or  
205 sensitivity to environmental conditions. We chose to evaluate the potential impact of an increased  
206 rearing water temperature during gametogenesis on rainbow trout spermatozoa methylome. To this  
207 aim, we analyzed by WGBS the sperm of 6 fish bred at 16°C from May to November versus 6 control  
208 fish kept at 12°C (**Tab 1**, **Suppl Fig 1B**). Global average methylation rates were similar in both conditions  
209 (**Suppl Tab 1**) and the PCA analysis did not show any strong segregation between 12°C and 16°C  
210 spermatozoa samples (**Fig 4A and Suppl Fig 2**). Nevertheless, we identified 112,386 DMCs with a  
211 threshold adjusted p-value of 0.05 (**Fig 4B**). Nearly 50% of DMCs were hypomethylated (55,738) and  
212 50% hypermethylated (56,648) at 16°C compared to 12°C. Interestingly, we found that 92% of these  
213 DMCs fall into differentially methylated regions (DMRs). This result indicates that an increase of rearing  
214 water temperature during gametogenesis alters spermatozoa methylome in a non-sporadic and non-  
215 random way. The affected cytosines instead concentrate into 5,359 DMRs of 400 bp median length  
216 resulting from a coordinated response to temperature increase. Ten % of these DMRs affect  
217 promoters, later on designed as prom-DMRs. Noteworthy, very few of these differentially methylated  
218 promoters had been previously identified as tissue specific, i.e. hypomethylated exclusively in either

219 spermatozoa or muscle (11 and 7, respectively) (**Fig 5A**). On the contrary, they mostly fall in the  
220 common hypo-, common hyper- or common moderately-methylated promoter categories, already  
221 indicating that developmental genes epigenetically reprogrammed in the spermatozoa were mostly  
222 unaffected.

223 In order to thoroughly study the biological functions of the differentially methylated promoters (prom-  
224 DMRs), we carried out both (i) a non-biased search for GO terms enrichment, (ii) a manual examination  
225 and annotation of the TOP 200 differentially methylated ones. Both approaches were mainly based on  
226 gene orthologies and mammalian literature. Examples of hypo- and hyper-methylated gene promoters  
227 and enriched GO categories are indicated in **Fig 5A**, **Suppl Tab 4 A-B** and **Suppl Tab 5**. Interestingly, the  
228 manual annotation analysis largely confirmed the GO term enrichment test and indicated that the  
229 prom-DMRs impacted spermiogenesis and lipid metabolism genes, leaving most developmental genes  
230 unaffected.

231

### 232 *Intracellular vesicle trafficking*

233 We found prom-DMRs annotations covering virtually the whole spectrum of the molecular  
234 components regulating vesicular trafficking and/or autophagy: from plasma and organelle membrane  
235 regulation to motor proteins and signaling (**Fig 5A**, **Suppl Tab 5**). We found DMRs in the promoters of  
236 *asah2* (N-AcylSphingosine AmidoHydrolase) (**Fig 5B**), *ppm1la* and *cert1* (ceramide transporters), *sphk1*  
237 (sphingosine kinase) and *mdga1* (MAM Domain containing Glycosylphosphatidylinositol Anchor),  
238 which regulate membrane ceramid and sphingolipid metabolism, therefore affecting membrane  
239 properties, lipid rafts, protein sorting, signaling, plasma membrane curvature and endocytosis  
240 initiation (Pepperl et al., 2013; Tani et al., 2007; Young and Wang, 2018). The promoter of the  
241 membrane receptor *s1pr3* (protein-G-coupled receptor of Phospho-Sphingosine) is differentially  
242 methylated as well as the promoters of *vldlr* and *mesd* (very low density lipoprotein receptor and  
243 chaperone for low density lipoprotein receptors (LRP), respectively), likely affecting endocytosis

244 initiation. We found several promoters of signaling molecules regulating vesicular traffic such as *grb2b*,  
245 *praf2* and *sh3gl1b* (adaptors), *rab1b* and *rnd2* (small GTPases), *dennd5a*, *rgl2*, *arhgef18a*, *quo*, *fgd4b*  
246 and *trappc13* (GEF, guanine nucleotide exchange factors for small GTPases), and *git2a*, *tbc1d2* and  
247 *rgs3b* (GAP, GTPases activating proteins). DMRs also affected promoters of vesicular traffic effectors  
248 such as the motor myosin *myo5b* (**Fig 5B**), the actin interacting WASP family member *wasf3a* and the  
249 kinesin *kif26ba*. Finally, we found prom-DMRs potentially affecting the regulation of membrane fusion,  
250 such as the promoters of *nsfl1c* (*p47* ortholog) and *nsfb* (*nsf* ortholog) (**Fig 5B**) encoding representative  
251 actors of the two major membrane fusion pathways (NSF and VCP/p47 pathways) (Brunger and  
252 DeLaBarre, 2003), the promoter of *rnf126* (E3 ubiquitin ligase, which is essential for proteasomal  
253 degradation in the VCP/p47 pathway) (**Fig 5B**), or the promoter of *myofl* involved in membrane fusion  
254 and repair. Markers of various types of intracellular vesicles were found differentially methylated,  
255 such as *ap5b1* and *ap5s1*, encoding two subunits of the late endosome AP-5 adaptor complex,  
256 *trappc13* encoding a component of the trafficking particle protein complex and *vmp1* (vacuole  
257 membrane protein), both involved in secretion and autophagy, therefore suggesting that several  
258 pathways orchestrating cellular trafficking might be elicited in response to increased rearing  
259 temperature. Therefore, sperm cells exposed to increased temperature exhibit a differential  
260 methylation at numerous promoters regulating vesicular trafficking potentially involving protein  
261 folding and degradation, cell signaling, autophagy or apoptosis.

262

### 263 *Cytoskeleton remodeling*

264 We found abundant prom-DMRs indicative of a reorganization of the cytoskeleton, mostly affecting  
265 actin network, cell-cell and cell-extra cellular matrix (ECM) adhesion (**Fig 5A, Suppl Tab 5**). As such, we  
266 found promoters of genes controlling actin dynamics: *flna* (filamin a) (**Fig 5B**), *tmod1* (tropomodulin)  
267 (**Fig 5B**), *add2* (adducin), *coro1b* (coronin) and *prkd4* (kinase) (**Fig 5B**), are all reported to interact with  
268 actin network and/or control its assembly, filament elongation or depolymerization. In addition,

269 *sema4gb*, encoding a semaphorin was found among the prom-DMRs (**Fig 5B**). Semaphorins regulate  
270 intra-cellular actin filaments and microtubule network interactions with the ECM. Interestingly, in the  
271 same pathway, the promoters of the semaphorin receptors *plkdc2* (plexin) and *nrp1a* (neurpilin) and  
272 its co-factor *rftn2* (raftlin) were also differentially methylated. Other promoters of genes encoding  
273 components of cellular junctions or cell-ECM adhesion complexes were found among DMRs, such as  
274 the protocadherins *pcdh1g32* and *pcdhb*, and the cadherin interacting angiomin-like *amotl2a*  
275 (adherens junctions), *nectin1a* (adherens junctions), the claudin encoding *clnd* and *clnd23a* (tight  
276 junctions). Finally, other prom-DMRs were reported in the literature to be regulators of cell-cell and  
277 cell-ECM adhesion, such as *susd2*, *mpp3b*, *sh3bgrl*, *tbc1d2*, *mdga2a*, *cadm1b* and *rxylt1*. These results  
278 highlight a high occurrence of genes related to actin cytoskeleton remodeling, cell-cell and cell-ECM  
279 adhesion, among promoters affected by an increased temperature in spermatozoa. This suggests a  
280 potential response of differentiating germ cells at the level of both intra- and inter-cellular processes.

281 Therefore, numerous prom-DMRs annotations converge toward a functional adaptation of germ cells  
282 to a temperature stress with a coordinated cytoskeletal reorganization supporting an important  
283 modulation of intracellular vesicular trafficking and autophagy. This could be interpreted as a  
284 conserved and generic cytoprotective response to heat stress. Similar results were indeed reported by  
285 Madeira *et al.* who observed that larvae of sea bream *Sparus aurata* modulate, at the protein level,  
286 the intracellular transport, protein folding and degradation and the cytoskeleton dynamics pathways  
287 in response to temperature stress (Madeira *et al.*, 2016). Indications of cytoskeleton remodeling in  
288 adaptation to increased temperature were also observed in a blue mussel gills (Fields *et al.*, 2012) or  
289 in the myofibril tissue of the crab *P. cinctipes* (Garland *et al.*, 2015). During their progression, male  
290 germ cells in rainbow trout thus modulate promoters classically involved in somatic heat stress  
291 adaptation and keep traces of these alterations in the terminally differentiated spermatozoa.

292

293 *Spermiogenesis*

294 Beyond the activation of possibly pan cell type adaptive mechanisms to increased rearing temperature,  
295 the cellular trafficking and cytoskeleton actors differentially methylated in our study interestingly  
296 pinpoint one major function precluding the spermatozoon stage: spermiogenesis. In the testis,  
297 differentiating sperm cells undergo drastic cell shape and cell-cell adhesion remodeling. These  
298 processes result from a tight control of the cytoskeleton dynamics and a recycling of intercellular  
299 junctions and ECM-interacting receptors at the plasma membrane, processes which are in turn  
300 dependent on endocytosis, vesicle trafficking, autophagy and protein recycling. Indeed, proper Golgi  
301 orientation, cytoskeleton, membrane fusion and vesicular trafficking are essential for the formation of  
302 the acrosome. Rainbow trout spermatozoa possess a transitory pseudo-acrosomal vesicle which is  
303 visible at the round spermatid stage and only leaves a cytoskeletal scar at later stages, reminiscent of  
304 mammalian acroplaxome (Billard, 1983). Endocytosis and vesicle trafficking are also crucial to the  
305 intraflagellar vesicle transport of molecules allowing the growth of the flagellum, and later on, for the  
306 elimination of the cytoplasm in late spermiogenesis and the individualization of sperm cells from the  
307 syncytium for spermiation. These spermiogenetic processes might recruit and coordinate common  
308 actors of vesicular trafficking expressed during spermiogenesis. It is therefore tempting to speculate  
309 that several gene promoters instrumental for spermiogenesis have been targeted by differential  
310 methylation. Interestingly, we found *tmf1* in the list of differentially methylated promoters (**Fig 5B**). Its  
311 mouse ortholog TMF/ARA160 encodes a protein associated to Golgi, emerging vesicles and  
312 microtubule network, which KO in male germ cells induces Golgi misorientation, lack of homing of pro-  
313 acrosomal vesicles, agenesis of the acrosome, inefficient cytoplasm removal and misshapen sperm  
314 head (Elkis et al., 2015; Lerer-Goldshtein et al., 2010). Myosin VI and Myosin Va, orthologs of *myo5b*,  
315 which we found differentially methylated in our data set, are essential for acrosome formation in the  
316 mouse and in the Chinese mitten crab (*E. sinensis*), respectively (Sun et al., 2010; Zakrzewski et al.,  
317 2020). The small GTPase *rnd2* mentioned earlier (“intracellular vesicle trafficking section”) was  
318 detected in Golgi and pro-acrosomal vesicles of rat spermatids (Naud et al., 2003).

319 In addition, several promoters of genes specifically implicated in other aspects spermatogenesis were  
320 found differentially methylated. As such, *rad51ap1* (**Fig 5B**), *rec8b* and *rmi2* (**Fig 5B**) encode essential  
321 actors of the meiotic homologous recombination (Bommi et al., 2019; Dray et al., 2011; Pires et al.,  
322 2017; Velkova et al., 2021), *aurka* encodes the aurora A kinase, involved in centrosome maturation  
323 and spindle formation during mitosis and also found to be essential for meiosis and spermiogenesis in  
324 mice (Lester et al., 2021), and *mief* (**Fig 5B**) encodes in human a mitochondrial fusion factor (Zhao et  
325 al., 2011), reminiscent of the mitochondrial remodeling to which rainbow trout spermatozoa are  
326 subjected during their differentiation. Interestingly, while autophagy is involved in spermiogenesis,  
327 and in particular in acrosome formation in mice (Wang et al., 2014), its increase in the human  
328 pathological model of cryptorchidism is associated to an impairment of spermatozoa maturation  
329 (Yefimova et al., 2019). Remarkably, in spite of the wide variation in spermatozoa morphologies and  
330 action modes among animals, cytoskeleton remodeling, vesicle trafficking and autophagy seem to be  
331 part of common pathways regulating normal, adaptive or pathological male germ cell maturation  
332 (White-Cooper and Bausek, 2010). Prom-DMRs which we detected in our experimental contrast are  
333 enriched in these GO categories. Altogether, these results argue in favor of an altered regulation of  
334 the late spermatogenic program (meiotic and post-meiotic) upon increased temperature. This could  
335 suggest that regions of the genome which were active during the exposure of the fish to the  
336 temperature stress (orchestrating late spermatogenesis) were more particularly prone to undergo a  
337 modulation of their methylation level.

338

### 339 *Lipid metabolism*

340 Interestingly, a third category of abundant prom-DMR annotations relates to the regulation of lipid  
341 metabolism (**Fig 5A, Suppl Tab 5**). We found several promoters of lipid catabolism genes such as *asah2*  
342 (ceramidase), *daglb* (lipase) or *abcd3a* (encoding a transporter involved in peroxisomal transport or  
343 catabolism of very long chain fatty acids). The regulation of long chain fatty acid metabolism was more

344 specifically affected with the differential promoter methylation of *cox-1* (cyclooxygenase involved in  
345 arachidonic acid conversion) (**Fig 5B**), *ptges* (terminal enzyme of the cox-2-mediated prostaglandin E2  
346 biosynthesis from arachidonic acid), *gstp1* (glutathione S-transferase), *gpx1* (glutathione peroxidase)  
347 and *elovl7b* (fatty acid elongase) (**Fig 5B**). In addition, the promoter of *gps2* (**Fig 5B**), which encodes a  
348 PPAR $\gamma$  transcriptional co-activator, was found differentially methylated. Fatty acid composition in  
349 testis was shown to be of particular relevance for sperm maturation and function in human (Collodel  
350 et al., 2020), in ruminant (Van Tran et al., 2017), or rooster (Teymouri Zadeh et al., 2020). PPAR $\gamma$ ,  
351 known to be master regulator of adipocyte differentiation and lipid metabolism, was also shown to  
352 control fatty acid metabolism in human and therefore suspected to be instrumental for proper sperm  
353 formation (Olia Bagheri et al., 2021). Finally, the promoter of *hnf4a*, which controls fatty acid oxidation  
354 and metabolism in hepatocytes, was overlapping with a DMR in our analysis (**Fig 5B**). The appearance  
355 of fatty acid metabolism as a potentially altered pathway, controlled by PPAR $\gamma$  and/or Hnf4a, could be  
356 interpreted as a metabolic adaptation of poikilotherm fish to temperature. The activity of several  
357 desaturases, elongases and fatty acid composition where indeed shown to be sensitive to temperature  
358 in fish and rainbow trout in particular (Tocher et al., 2004). It is remarkable however to find its scar in  
359 the methylation profile of spermatozoa.

360

## 361 **CONCLUSION**

362 In the present study, we aimed at characterizing the rainbow trout sperm methylome (using muscle as  
363 a somatic reference tissue) and its sensitivity to a rearing temperature increase of 4°C during  
364 spermatogenesis. Single-base resolution methylomes revealed that an important pool of gene  
365 promoters is methylation-free in spermatozoa, and therefore in a permissive state for transcription,  
366 although sperm DNA is tightly packed and transcriptionally silent. Sperm cells thus carry an epigenetic  
367 code seemingly resulting from history and preparing their future role. Remarkably, sperm methylation-  
368 free promoters control housekeeping, early development and germline functions.



369 We found that an increased rearing temperature during spermatogenesis significantly impacted sperm  
370 methylome. Interestingly, DMRs affected promoters controlling spermiogenesis and lipid metabolism  
371 genes, leaving most developmental genes unaffected. This organized response to heat stress suggests  
372 a coordination by a signaling pathway. Our data highlighted PPAR $\gamma$  and Hnf4 pathways. Alternatively,  
373 some genomic regions might be more prone to undergo methylome alterations because of a pre-  
374 established sensitivity, laying in the fact that they are active during spermiogenesis, which is both a  
375 period of deep chromatin remodeling and the time of experimental stress exposure. In an attempt to  
376 identify a potential driver of the differential methylation we observed, we looked for enriched  
377 transcription factor binding sites (TFBS) in our DMR using MEME suite. Interestingly, the most  
378 significantly enriched TFBS was the one of PRDM9 (**Suppl Fig 3**), which binds to recombination hotspots  
379 during meiosis. This opens the seducing possibility that meiotic recombination sites are at particular  
380 risk of epigenetic reprogramming upon environmental stress. Taken together, for the first time in  
381 rainbow trout, our results demonstrate that the methylation status of sperm-specific gene promoters  
382 controlling housekeeping and developmental function is very robust in the context of a 4°C  
383 temperature increase during spermatogenesis. Remarkably however, we found that the sperm  
384 methylome is altered and carry the traces of the life history of the individuals. We found epigenetic  
385 alterations of spermiogenesis and lipid metabolism controlling genes, which will be transmitted to the  
386 next generation. Future investigation should answer whether this altered sperm epigenome could be  
387 a molecular basis of acclimation to a heat stress for next generations and whether it could impact  
388 positively or negatively offspring performances under various conditions.

389

390

391

392

## 393 MATERIAL AND METHODS

### 394 Ethics statements

395 The experiment was conducted following the Guidelines of the National Legislation on Animal Care of  
396 the French Ministry of Research (Decree No 2013–118, February 2013) and in accordance with EU legal  
397 frameworks related to the protection of animals used for scientific purposes (i.e., Directive  
398 2010/63/EU). The scientists in charge of the experiments received training and personal authorization.  
399 The experiment was conducted at the INRAE Physiology and Genomic Laboratory (LPGP) experimental  
400 facilities (permit number C35-238-6, Rennes, France), and approved by the ethics committee for the  
401 animal experimentation of Rennes under the authorization number T-2019-05-AL.

402

### 403 Fish rearing and sampling

404 *Oncorhynchus mykiss* rainbow trouts, from a winter spawning strain (December 15 – January 15), were  
405 reared in our experimental farm (river water, in which temperatures are near 7.5°C in winter and can  
406 reach 20°C in summer). Two groups of 20 males were transferred to our indoor fish facility in April  
407 (April 4<sup>th</sup> 2019) of their second year, when they reached 15 months of age. They were kept in a recycling  
408 system, under artificial spring/summer/autumn photoperiod, and at the temperatures of 12°C and  
409 16°C (after 2 weeks acclimation and gradual temperature increase, from April 18<sup>th</sup> 2019 till sample  
410 collection). Regarding the reproductive cycle, this period of experimental exposure corresponds to the  
411 post-meiotic phase. For each experimental group, fish were split into two water tanks (10 fish per  
412 tank). The growth of the fish in both groups was recorded and their commercial diet rations were  
413 adapted so that both groups follow similar growth curves (Supplementary Figure 4). Fish were checked  
414 every two weeks for milt production, at the occasion of the measure of their individual weight. Sperms  
415 were harvested by stripping (first spermiation) and muscle samples were taken after euthanasia  
416 (tricaine 200 mg/mL).

## 417 **Whole genome bisulfite sequencing**

418 We analyzed by WGBS and compared (i) the sperm of 4 fish bred at 12°C *versus* the muscle of the same  
419 4 fish (reared in control condition), and (ii) the sperm of 6 fish bred at 16 °C from mid-April to November  
420 *versus* 6 control fish kept at 12°C (**Tab 1, Suppl Fig 1B**). Genomic DNA was prepared after an overnight  
421 lysis in TNES/Urea buffer (10 mM Tris, 0.125 M NaCl, 10 mM EDTA, 17 mM SDS, 4M urea, pH 8)  
422 complemented with 80ug/mL proteinase K at 42°C. After phenol-chloroform extraction, the samples  
423 were treated with RNase A (4mg/mL) and gDNAs were precipitated by the addition of isopropanol and  
424 sodium acetate. Whole genome bisulfite sequencing libraries were prepared (according to Accel-NGS  
425 Methyl-Seq DNA library Kit for Illumina protocol, Swift Biosciences) and sequenced in 150 bp paired-  
426 end reads by Novogene company.

427

## 428 **Bio-informatic analysis**

429 WGBS yielded a minimum of 300 million paired-end 150 bp reads per sample (Tab 1). Quality and  
430 adapter trimming of the reads was carried out using Trim Galore (with an additional 10 bp clipping for  
431 read2 5'-end and read1 3'-end) (Krueger and Galore, n.d.). After trimming, only uniquely mapped reads  
432 on the *O. mykiss* genome or its *in silico* bisulfite converted version were kept for further analysis (using  
433 Bismark bio-informatic software and Omyk\_1.0 genome version, (Krueger and Andrews, 2011)).  
434 BigWig files were obtained with bedGraphToBigWig (Kent et al., 2010) and methylation values were  
435 visualized with Integrative Genome Viewer (Robinson et al., 2011). These steps were included in a  
436 workflow which agrees to FAIR principles and is accessible online  
437 (<https://forgemia.inra.fr/lpgp/methylome>). The methylome (v1.0) workflow was built in Nexflow dsl2  
438 language using a singularity container and optimized in terms of computing resources (cpu, memory)  
439 for its use on an informatic farm with a slurm scheduler.

440 Differentially methylated cytosines (DMCs) were identified by the R package DSS (Feng et al., 2014;  
441 Wu et al., 2015), using the optional methylation level smoothing on 500 bp and a Benjamini-Hochberg  
442 adjusted p-value threshold of 0.05. Differentially methylated regions (DMRs) were defined using DSS  
443 package with seed regions containing at least 5 CpGs and expansion window criterias agreeing with a  
444 minimum of 75 % of DMCs (with raw pval < 0.01).

445 CpGs were annotated with GenomeFeatures (Brionne, n.d.), RepeatMasker (Smit, 2015) and ensembl  
446 v105.1 annotation. Biological interpretations were carried out using ViSEAGO R package (Brionne et  
447 al., 2019) and the Gene Ontology (GO) public database. Associated gene terms were implemented  
448 from the extended Ensembl Compara custom annotation based on the release 103. Enrichment tests  
449 were performed using exact Fisher's test. Enriched GO terms (p-val < 0.01) were grouped into  
450 functional clusters by Wang's semantic similarity respecting GO graph topology and Ward's criterion.

451

#### 452 **AVAILABILITY OF DATA**

453 The data that support the findings are already deposited and will be available on the ENA archive  
454 (<https://www.ebi.ac.uk/ena/browser/home>) under the accession number (PRJEB56697) and following  
455 an embargo from the date of publication. They can be transferred to the reviewers upon editor  
456 request.

457

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463

464 **AUTHORS CONTRIBUTIONS STATEMENT**

465 AL designed the project and experiments, supervised the study and wrote the manuscript. CL and JLL  
466 contributed to the design of the project. ASG, SK and CL performed the wet lab work. AB performed  
467 the bio-informatic analysis. All the authors read and approved the manuscript.

468

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Tissue	Fish	Total raw reads	Trimmed reads	% of mapping	BS conversion (%)	Nuclear					Mitochondrial	
						Average CpG sequencing depth (x)	% CpGs covered 1x	% CpGs covered 5x	% CpGs covered 10x	CpG methylation (%)	Average CpG sequencing depth (x)	CpG methylation (%)
Muscle 12°C	1	324 054 890	312 036 061	60,16	99,35	11,02	93,38	78,93	54,04	78,13	2152,99	1,03
	2	314 187 112	301 636 081	58,86	99,38	10,46	92,47	77,44	50,38	74,80	2122,71	0,95
	3	323 609 587	313 382 478	60,34	99,36	11,24	92,59	78,67	54,56	74,47	2534,55	0,78
	4	346 904 275	334 318 944	60,55	99,37	12,05	93,06	79,82	58,62	75,70	3314,62	0,75
				59,98	99,37	11,19	92,88	78,71	54,40	75,77	2531,22	0,88
Sperm 12°C	1	300 587 067	294 303 143	59,66	99,15	10,58	92,29	77,88	50,85	88,41	74,09	7,65
	2	325 691 293	321 065 565	61,02	99,42	11	92,52	78,78	54,54	88,32	77,54	6,98
	3	336 626 182	332 307 052	61,31	99,39	11,54	92,57	79,35	56,11	88,47	62,51	5,23
	4	309 900 540	305 834 179	46,18	99,48	8,97	92,11	71,22	37,33	88,74	75,62	5,66
	5	332 585 217	326 170 573	59,67	99,50	11,07	92,76	78,98	54,21	88,25	39,75	11,90
	6	306 207 661	301 165 523	60,48	99,38	10,68	92,29	77,88	50,85	88,49	29,34	9,80
				58,05	99,39	10,64	92,43	76,81	49,71	88,45	59,81	7,87
Sperm 16°C	7	340 298 820	335 329 836	61,66	99,39	11,14	92,77	79,58	56,22	88,43	34,56	15,56
	8	309 013 645	304 553 951	60,67	99,40	10,71	92,71	78,56	52,32	88,71	113,23	5,21
	9	313 593 505	307 126 184	60,35	99,34	10,81	92,68	78,64	52,58	88,46	123,90	4,62
	10	311 388 841	305 315 136	61,97	99,35	11,17	92,51	78,71	54,59	88,77	76,70	4,12
	11	336 389 118	330 578 540	60,27	99,40	11,37	92,64	79,30	55,42	88,62	74,01	7,00
	12	311 912 118	306 606 582	60,73	99,36	11,03	92,59	78,91	54,24	88,61	54,91	5,06
				60,94	99,37	11,04	92,65	78,95	54,23	88,60	79,55	6,93

**Tab 1. Summary of WGBS**

Paired reads were mapped uniquely to the rainbow trout genome (Omik\_1.0) using Bismark. Average sequencing depth. indicates the mean number of reads per position in nuclear and mitochondrial genomes. The nuclear coverage represents the proportion of mapped CpGs over the total number of CpG sites in the nuclear genome, here calculated under 1X, 5X or 10X sequencing depth selection threshold.

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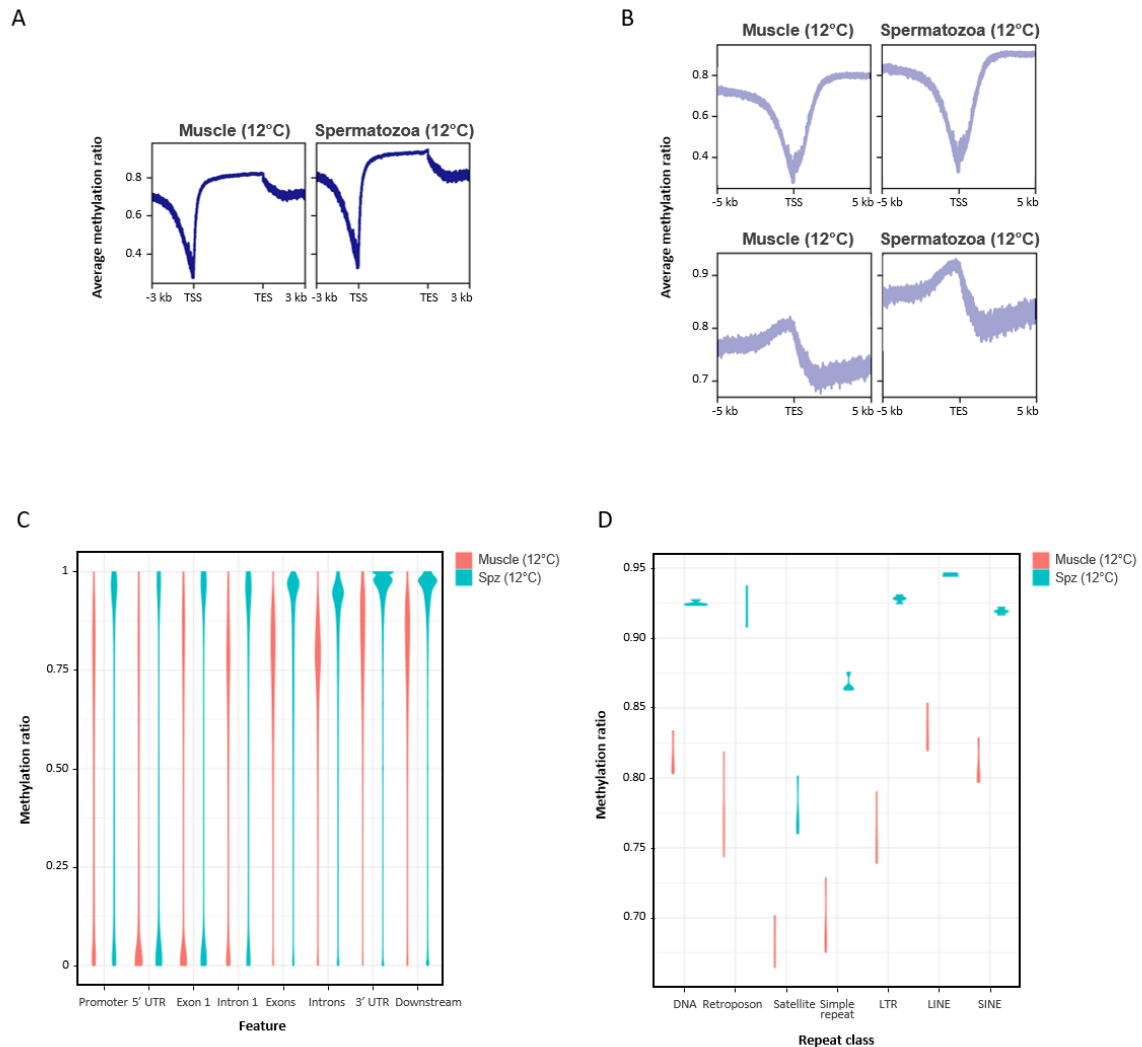
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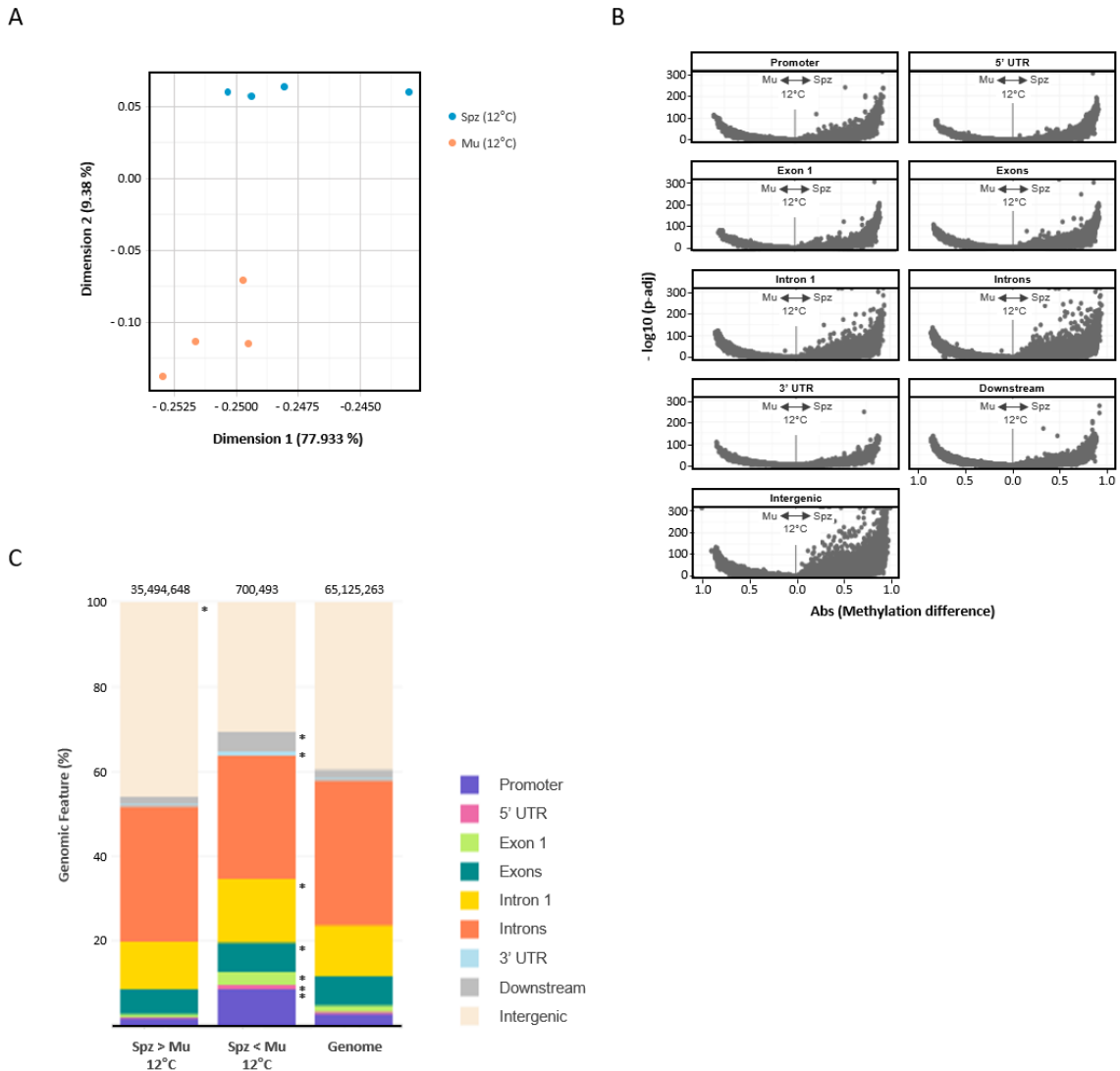
646 **Fig 1. Global DNA methylation in rainbow trout control muscle and spermatozoa according to**  
 647 **genomic features**

648 A. Average methylation profiles along the 115 853 annotated ensembl coding and non-coding  
 649 transcripts of ensembl v104 Omyk\_1.0 genome. B. Average methylation profiles around TSS (+/- 5 kb)  
 650 (left panels) and TES (+/- 5 kb) (right panels). C. Distribution of methylation ratios according to gene  
 651 features: promoter regions (-1 kb ; TSS), 5'UTR, exon1, intron1, all exons, all introns, 3'UTR and  
 652 downstream regions (TES ; +1 kb). D. Distribution of methylation ratios found in muscle and sperm in  
 653 transposons (DNA), retroposons, satellites, simple repeats, LTR containing elements, LINES and SINES.

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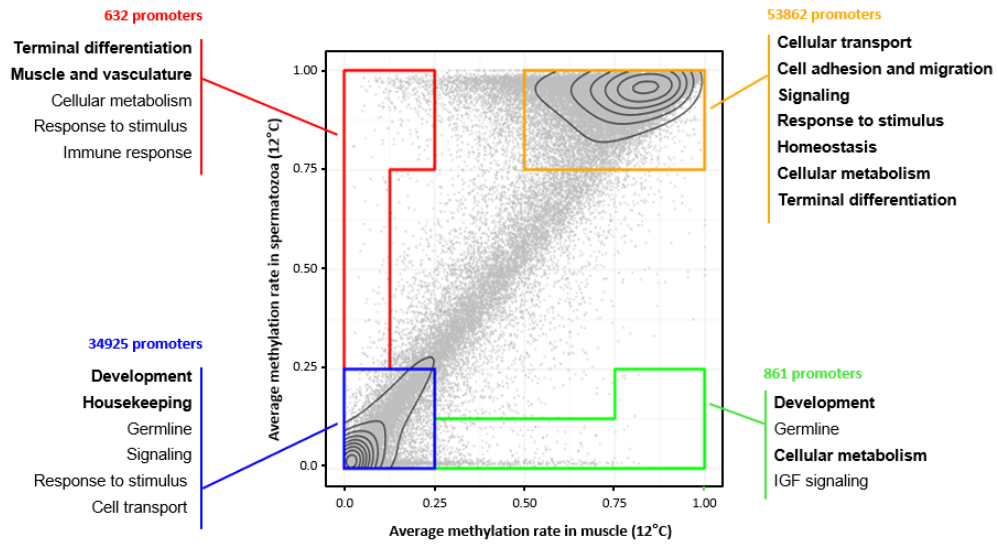
## 659 Fig 2. Differentially methylated cytosines between control spermatozoa and muscle

660 A. PCA plot of spermatozoa and muscle WGBS data sets. B. Volcano plots of DMCs between  
 661 spermatozoa and muscle in every genomic feature (x axis : absolute methylation difference, y : -log<sub>10</sub>  
 662 (adjusted p-value)). C. Relative distributions of DMCs (Spz > Mu : hypermethylated in sperm  
 663 compared to muscle; Spz < Mu : hypomethylated in sperm compared to muscle) according to  
 664 genome features (promoter regions (-1 kb ; TSS), 5'UTR, exon1, intron1, all exons, all introns, 3'UTR,  
 665 downstream (TES ; +1 kb) and intergenic regions). The reference set (Genome) contains all analyzed  
 666 CpGs. Genome features statistically enriched in DMCs compared to the reference set are indicated  
 667 with an \* (binomial test).

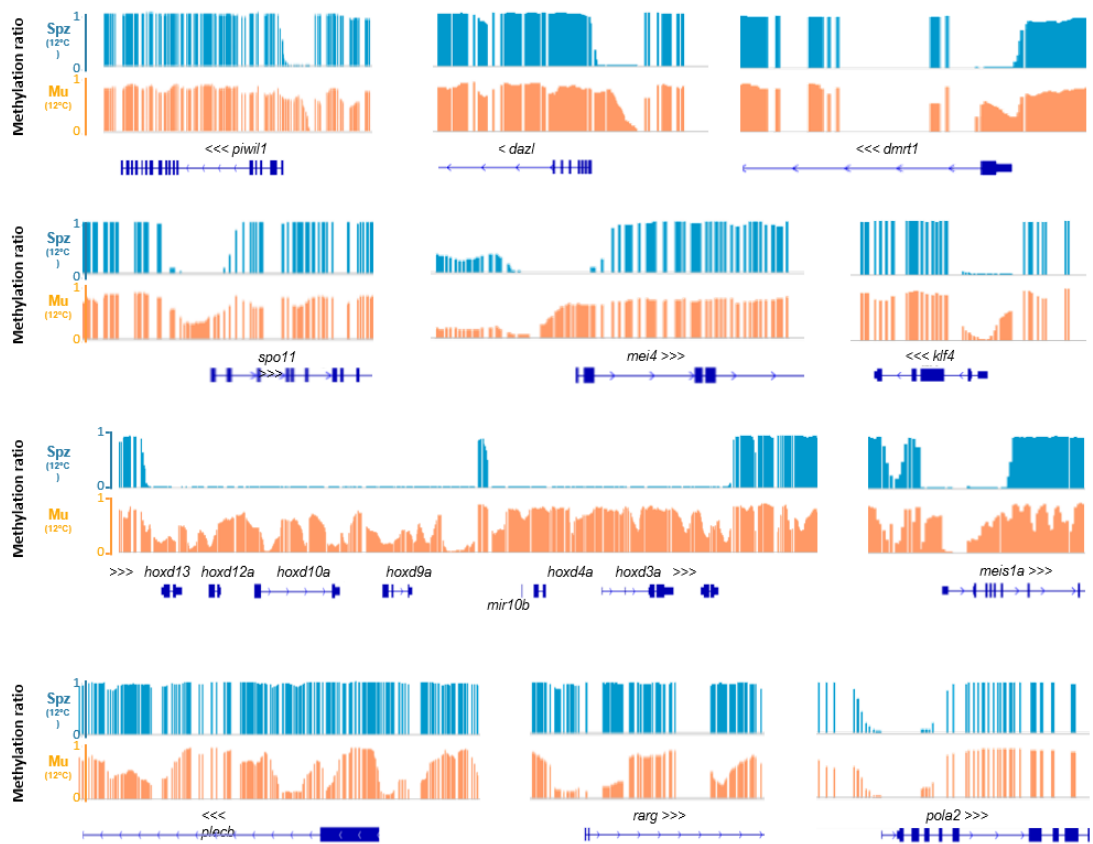
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673 **Fig 3. Differentially methylated gene promoters between control spermatozoa and muscle**

674 A. Plot of average methylation of ensembl omyk\_1.0 v105 transcripts promoters in muscle (x axis)  
675 versus spermatozoa (y axis). Common hypo-methylated promoters are highlighted in the blue  
676 square, common hyper-methylated promoters in the orange shape, muscle-specific hypo-  
677 methylated promoters in the red shape and spermatozoa-specific promoters in the green shape.  
678 Functional annotation terms deduced from a GO term enrichment analysis and corresponding to  
679 each class are indicated. B. IGB snapshots of transcripts or TSS regions as examples of spermatozoa-  
680 specific hypo-methylated promoters (*piwil1*, *dazl*, *dmrt1*, *spo11*, *mei4*, *hox* cluster and *mir10b*,  
681 *meis1a*), muscle-specific hypo-methylated promoters (*plecb* and *rarg*) or common hypo-methylated  
682 promoter (*pola2*). Muscle : Mu; Spermatozoa : Spz.

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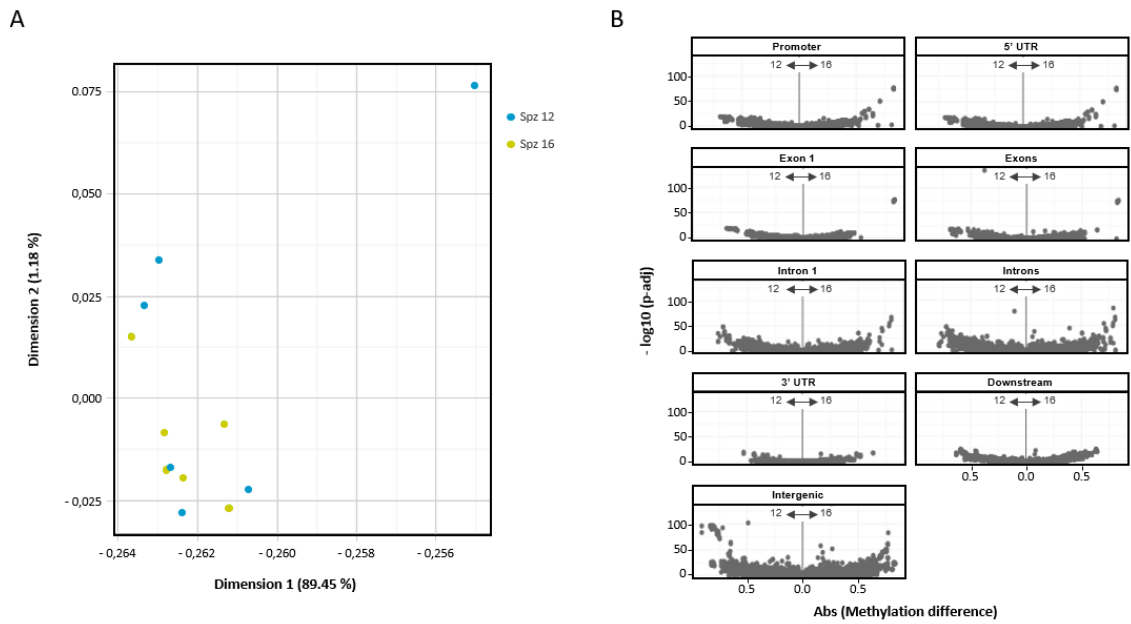
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701 **Fig 4. Differentially methylated cytosines between spermatozoa from males raised at 16°C versus**  
 702 **12°C**

703 A. PCA plot of spermatozoa from males raised at 12°C and 16°C WGBS data sets. B. Volcano plots of  
 704 DMCs between spermatozoa from males raised at 12°C versus 16°C in every genomic feature (x axis :  
 705 absolute methylation difference, y : -log10 (adusted p-value)).

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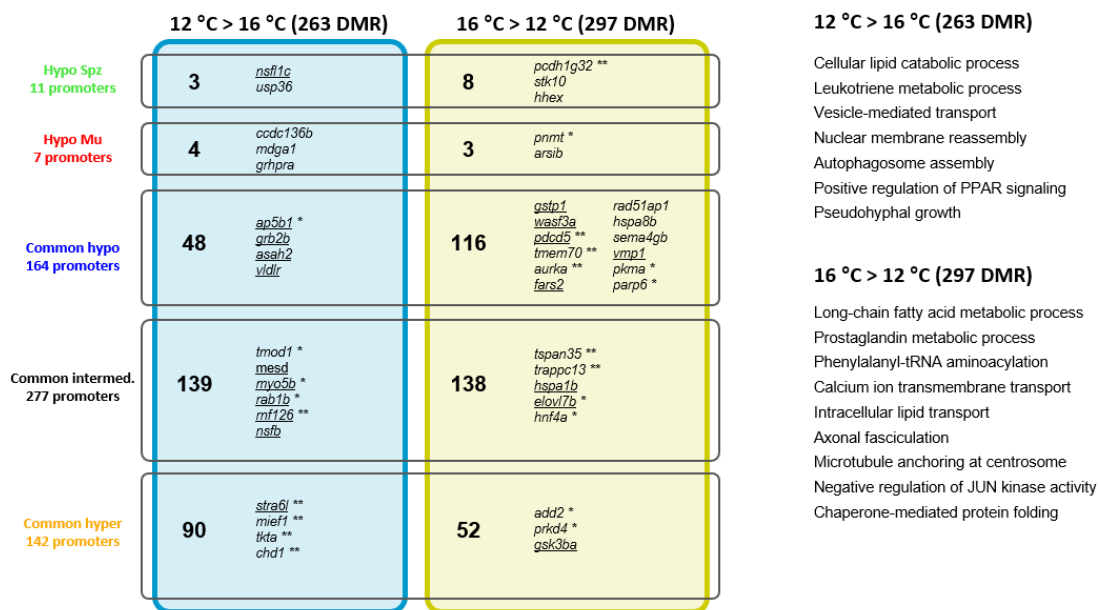
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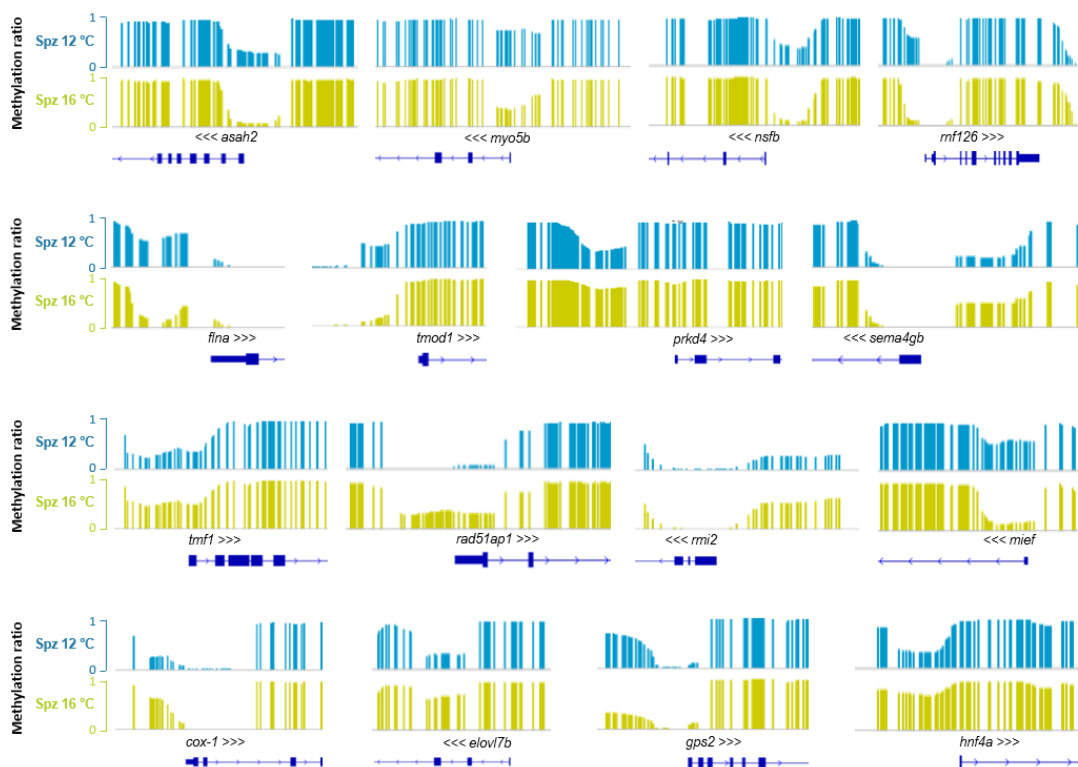
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719 **Fig 5: Differentially methylated gene promoters between spermatozoa of fish raised at 16°C versus**  
720 **12°C**

721 A. Intersection of up- and down-regulated DMRs with the different promoter categories defined in  
722 Fig 3 (sperm-specific hyper or hypo-methylated, equally methylated,...) and associated GO term  
723 enrichment analysis. Genes are given of examples of each categories, chosen either for their  
724 association to an enriched GO term or for their presence in the top200 list of prom-DMRs.  
725 underlined: associated to an enriched GO terms, \*: top 10, \*\*: top 50 prom-DMR. B. IGV snapshots of  
726 TSS regions as examples of hyper- and hypo-methylated promoters at 16°C versus 12°C.

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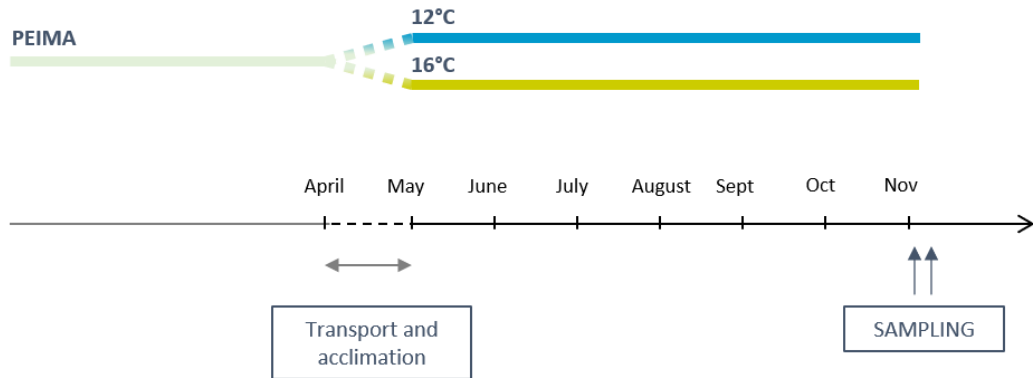
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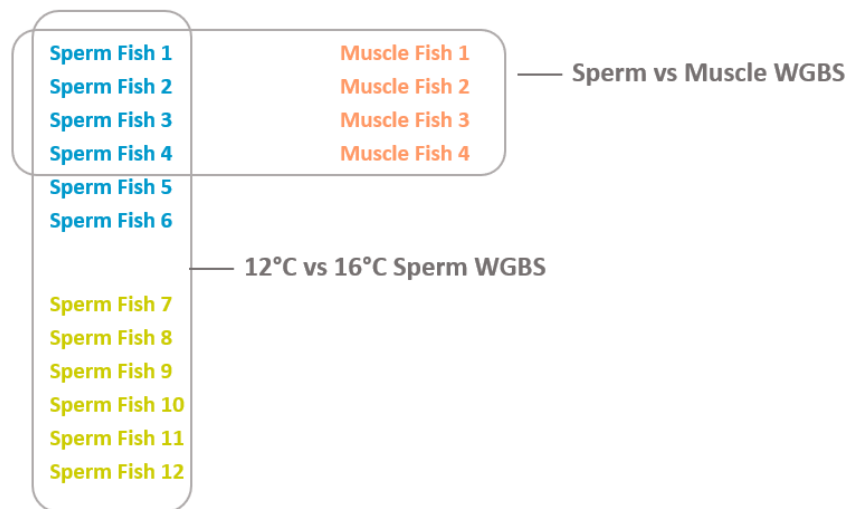
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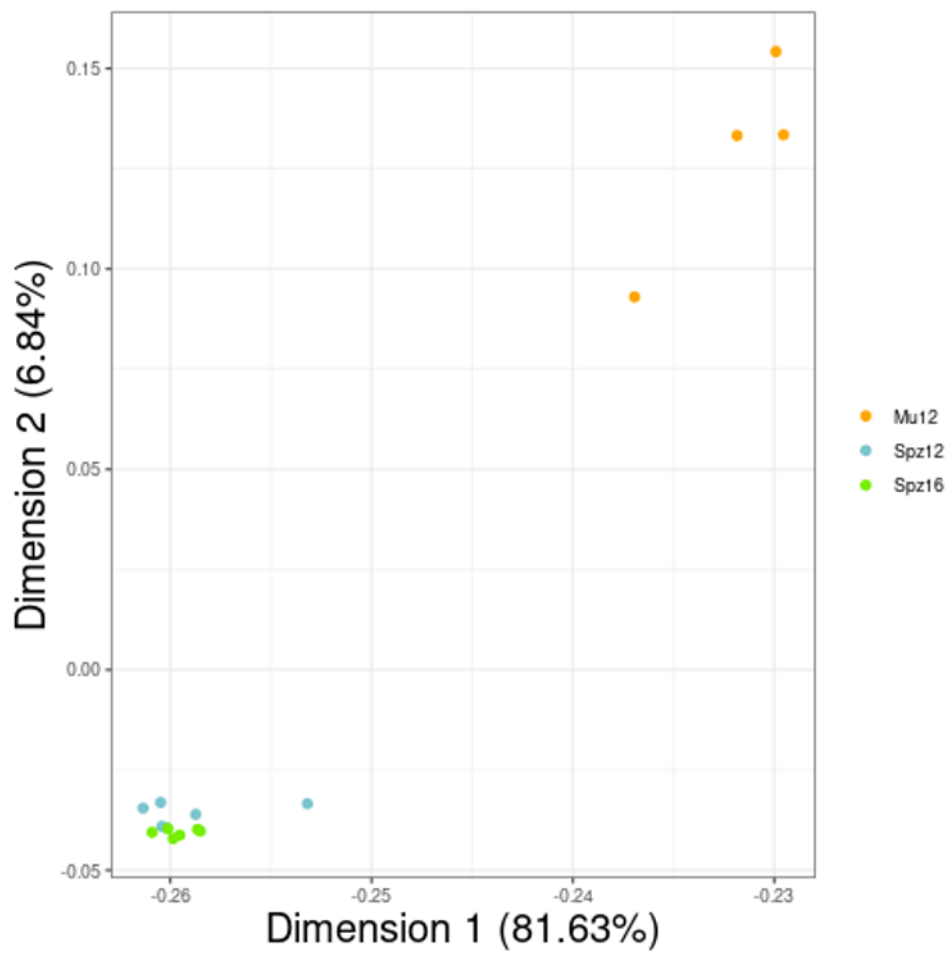
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748 **Suppl Fig 1. Experimental design**

749 A. *Oncorhynchus mykiss* rainbow trouts were reared in our experimental farm and transferred to our  
 750 indoor fish facility in April of their second year. Two groups of 20 males were kept in a recycling  
 751 system, under artificial spring/summer/autumn photoperiod, and at the temperatures of 12°C and  
 752 16°C (after 2 weeks acclimation and gradual temperature increase). Fish sperm and muscle were  
 753 sampled during the reproduction period. B. Four fish were sequenced for the sperm versus muscle  
 754 paired analysis while two groups of 6 fish were sequenced for the study of the temperature contrast  
 755 in spermatozoa.

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759 **Suppl Fig 2. PCA plot of muscle (Mu12), spermatozoa from males raised at 12°C (Spz12) and**  
 760 **spermatozoa from males raised at 16°C (Spz16) WGBS data sets**

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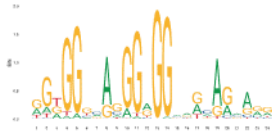
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PRDM9 p-val 9.28e-35



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769 **Suppl Fig 3. Putative recombination hotspots enrichment in DMRs between spermatozoa from**  
770 **males raised at 16°C versus 12°C.**

771 Vertebrate PRDM9 consensus DNA binding site identified by MEME in DMR sequences between  
772 spermatozoa from males raised at 16°C versus 12°C, and associated p-value.

773 MEME suite is accessible online (<https://meme-suite.org/meme/>), SEA algorithm was used.

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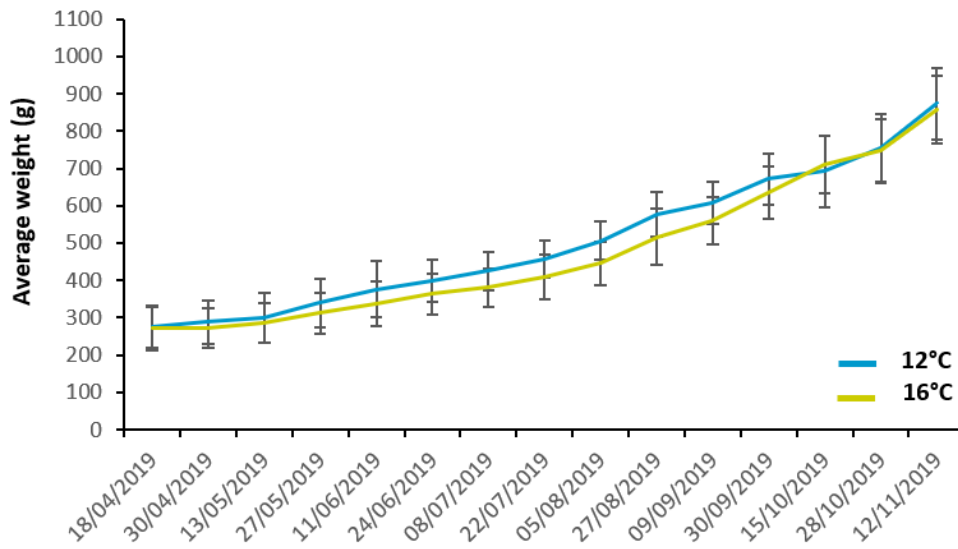
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### Weight of fish raised at 12°C and 16°C from April 18th to November 12th 2019



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791 **Suppl Fig 4. Evolution of the weight of the fish raised at 12°C and 16° during the exposure to the**  
792 **temperature increase.**

793 Fish weight was individually recorded every 2 weeks. The graph shows the weight averages and  
794 standard deviations found for each group between April 18 and November 12 2019.

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