Downloaded from orbit.dtu.dk on: Apr 27, 2024

DTU Library

Genome-wide methylation in the panmictic European eel (Anguilla anguilla)

Liu, Shenglin; Tengstedt, Aja Noersgaard Buur; jacobsen, Lars Magnus W.; Pujolar, Jose Martin; Jónsson, Bjarni; Lobón-Cervià, Javier; Bernatchez, Louis; Hansen, Michael Møller

Published in:
Molecular Ecology

Link to article, DOI:
10.1111/mec.16586

Publication date:
2022

Document Version
Peer reviewed version

Link back to DTU Orbit

Citation (APA):
Liu, S., Tengstedt, A. N. B., jacobsen, L. M. W., Pujolar, J. M., Jónsson, B., Lobón-Cervià, J., Bernatchez, L., & Hansen, M. M. (2022). Genome-wide methylation in the panmictic European eel (*Anguilla anguilla*). *Molecular Ecology*, *31*(16), 4286-4306. https://doi.org/10.1111/mec.16586

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

MOLECULAR ECOLOGY

Genome-wide methylation in the panmictic European eel (Anguilla anguilla)

Journal:	*Molecular Ecology*
Manuscript ID	MEC-22-0272.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	21-Jun-2022
Complete List of Authors:	Liu, Shenglin; University of Aarhus, Department of Biology Tengstedt, Aja; Aarhus University, Department of Biology Jacobsen, Magnus; Technical University of Denmark, National Institute of Aquatic Resources Pujolar, Jose Martin; Technical University of Denmark, National Institute of Aquatic Resources Jónsson, Bjarni; Institute of Freshwater Fisheries, Northern Division Lobón-Cervià, javier; National Museum of Natural Sciences (CSIC) Bernatchez, Louis; Université Laval, Biologie; Hansen, Michael; Aarhus University, Department of Biology;
Keywords:	Adaptive processes, Anguilla anguilla, epigenetics, hox clusters, Hybridization, methylation

SCHOLARONE™
Manuscripts

Genome-wide methylation in the panmictic European eel (*Anguilla anguilla*)

Shenglin Liu[1#], Aja Noersgaard Buur Tengstedt[1], Magnus W. Jacobsen[2], Jose Martin Pujolar[3], Bjarni Jónsson[4§], Javier Lobón-Cervià[5], Louis Bernatchez[6], Michael M. Hansen[1#]

[1] Department of Biology, Aarhus University, Aarhus, Denmark
[2] Section for Marine Living Resources, National Institute of Aquatic Resources, Technical University of Denmark, Silkeborg, Denmark
[3] Centre for Gelatinous Plankton Ecology and Evolution, National Institute of Aquatic Resources, Technical University of Denmark, Kongens Lyngby, Denmark
[4] North West Iceland Nature Center, Saudárkrókur, Iceland
[5] National Museum of Natural Sciences (CSIC), Madrid, Spain
[6] IBIS (Institut de Biologie Intégrative et des Systèmes), Université Laval, Québec, Canada

[#] Corresponding authors.
Shenglin Liu, Department of Biology, Aarhus University, Ny Munkegade 114-116, DK-8000 Aarhus C, Denmark. E-mail liushenglin1222@gmail.com
Michael M. Hansen, Department of Biology, Aarhus University, Ny Munkegade 114-116, DK-8000 Aarhus C, Denmark. E-mail mmh@bio.au.dk

[§] Current address: The Icelandic Parliament, Reykjavík, Iceland

For Review Only

Abstract

The role of methylation in adaptive, developmental and speciation processes has attracted considerable interest, but interpretation of results is complicated by diffuse boundaries between genetic and non-genetic variation. We studied whole genome genetic and methylation variation in the European eel, distributed from subarctic to subtropical environments, but with panmixia precluding genetically based local adaptation beyond single-generation responses. Overall methylation was 70.9%, with hypomethylation predominantly found in promoters and first exons. Redundancy analyses involving juvenile glass eels showed 0.06% and 0.03% of the variance at SNPs to be explained by localities and environmental variables, respectively, with GO terms of genes associated with outliers primarily involving neural system functioning. For CpGs 2.98% and 1.36% of variance was explained by localities and environmental variables. Differentially methylated regions particularly included genes involved in developmental processes, with hox clusters featuring prominently. Life stage (adult versus glass eels) was the most important source of inter-individual variation in methylation, likely reflecting both ageing and developmental processes. Demethylation of transposable elements relative to pure European eel was observed in European X American eel hybrids, possibly representing postzygotic barriers in this system characterized by prolonged speciation and ongoing gene flow. Whereas the genetic data are consistent with a role of single-generation selective responses, the methylation results underpin the importance of epigenetics in the life cycle of eels and suggests interactions between local environments, development and phenotypic variation mediated by methylation variation. Eels are remarkable by having retained eight hox clusters, and the results suggest important roles of methylation at hox genes for adaptive processes.

Key words:

Adaptive processes, *Anguilla anguilla*, epigenetics, hox clusters, hybridization, methylation.

For Review Only

Introduction

It is increasingly appreciated that epigenetics, defined as modifications of DNA that affects expression of genes but without changing the DNA sequence (Dupont *et al.* 2009), is highly important in developmental, adaptive and evolutionary processes (Adrian-Kalchhauser *et al.* 2020; Anastasiadi *et al.* 2021; Gore *et al.* 2018; Greenberg & Bourc'his 2019; Jablonka 2017; Jablonka & Raz 2009; Lind & Spagopoulou 2018; Stajic *et al.* 2019; Verhoeven *et al.* 2016). Epigenetics mechanisms include DNA methylation, histone modifications and small RNAs (Deans & Maggert 2015; Feil & Fraga 2012; Law & Jacobsen 2010), where DNA methylation has so far attracted the most interest. This involves addition of a methyl group to a nucleotide, in most cases Cytosin (C), which in animals primarily occurs at CpG sites (C followed by G in the genome sequence). Gain or loss of methylation at CpG sites in regulatory regions can lead to silencing or reactivation of genes, with hypomethylation of promoter regions generally leading to increased transcription in vertebrates (Christensen *et al.* 2021; Greenberg & Bourc'his 2019; Jones 2012; Law & Jacobsen 2010; Moore *et al.* 2013).

Methylation plays key roles in developmental processes and cell differentiation (Greenberg & Bourc'his 2019). In mammals this involves two events of reprogramming during embryogenesis, but general knowledge about major changes in methylation is scarce in other animals, including those that undergo extensive metamorphosis. Importantly, methylation can be environmentally induced and transferable across cell divisions (Feil & Fraga 2012). There is furthermore some evidence, particularly in plants but less clear-cut in animals that epigenetic marks can be transferable across generations. This can ultimately lead to traits being inherited despite not being coded by the DNA sequence (Anastasiadi *et al.* 2021; Gapp *et al.* 2014; Richards 2006; Schmitz *et al.* 2013; Skvortsova *et al.* 2018).

The environmental inducibility of methylation states raises the possibility that this could represent rapid adaptive mechanisms in response to spatial and temporal environmental variation (Angers *et al.* 2020; Feil & Fraga 2012), and it is assumed to be a major component in phenotypic plasticity and may also be involved in knock-on effects, that is early perceived environmental cues leading to phenotypic change later in life (Jonsson *et al.* 2022). Indeed, some studies report significant methylation differences of functional importance associated with environmental variation (Artemov *et al.* 2017; Gugger *et al.* 2016; Heckwolf *et al.* 2020; Le Luyer *et al.* 2017; Merondun *et al.* 2019; Metzger & Schulte 2018; Schmitz *et al.* 2013; Wogan *et al.* 2020) A distinction has usually been made between genetic adaptation (encoded by DNA) resulting from evolution across generations; and phenotypic plasticity, within-generation responses of individuals to environmental conditions, e.g. by adjustments in physiology (Gienapp *et al.* 2008; Kawecki & Ebert 2004). Whereas methylation from this perspective could be viewed as a source of non-genetic adaptation, it is increasingly realized that the boundaries between genetic and non-genetic factors are unclear and involve complex interactions, also in the case of epigenetics (Adrian-Kalchhauser *et al.* 2020; Taudt *et al.* 2016; Verhoeven *et al.* 2016). In particular, a proportion of methylation may be under genetic control (Richards 2006), as for instance demonstrated by different non-recombining chromosome inversions showing different methylation (Sun *et al.* 2021). Differences in methylation patterns between populations could therefore ultimately reflect individual- and population-level genetic differences in genes controlling methylation (Anastasiadi *et al.* 2021; Dubin *et al.* 2015; Richards 2006; Taudt *et al.* 2016). It would therefore be of significant interest to study epigenetic patterns and its association with geographical and environmental variation in species where genetically based adaptation can be ruled out.

For Review Only

Methylation also has the important role to repress transposable elements (TEs), thus preventing deleterious proliferation of TEs in the genome (Jones 2012; Slotkin & Martienssen 2007). Derepression of TEs by demethylation has been found to occur in some cases of hybridization, leading to harmful reactivation and proliferation of transposons (Laporte *et al.* 2019; Michalak 2009; O'Neill *et al.* 1998; Ungerer *et al.* 2006). This could potentially represent postzygotic barriers, but the importance of TE derepression relative to other postzygotic barriers remains unclear. It would therefore be of interest to study this in cases where environmental conditions experienced by species are similar and gene flow between species is still ongoing.

The European eel (*Anguilla anguilla*) represents an excellent model for further increasing our knowledge about the role of methylation in adaptive and developmental processes. It is distributed across environmental conditions spanning from subarctic climates in Iceland to Sub-tropical environments in North Africa (Tesch 2003). It spawns in the Southern Sargasso Sea in partial sympatry with its sister species American eel (*A. rostrata*) (Kleckner *et al.* 1983; Munk *et al.* 2010), which shows a very similar life history and is distributed along similar environmental gradients along the American Atlantic coast (Tesch 2003). European eel larvae are transported by ocean currents towards the European and North African coastal regions. The recently arrived juveniles metamorphose into so-called glass eels, settle in freshwater and coastal marine habitats and go through an additional stage of metamorphosis until they mature as silver eels and undertake their > 5,000 km spawning migration back to the Sargasso Sea (Schmidt 1923; Tesch 2003). Previous results based on anonymous methylation markers suggest major differences between life stages (Trautner *et al.* 2017), but it is unknown if these differences represent functionally important methylation in relation to developmental stages or merely ageing effects (Anastasiadi & Piferrer 2020; Horvath & Raj 2018).

Both European and American eel are remarkable by being panmictic species, that is despite being distributed across a wide range of environmental conditions they mate randomly in the Sargasso Sea (Als *et al.* 2011; Côté *et al.* 2013; Enbody *et al.* 2021; Palm *et al.* 2009; Pujolar *et al.* 2014b); though see contrasting views by e.g. Baltazar-Soares *et al.* (2014).Signals of spatially varying selection have been detected in both European and American eel (Babin *et al.* 2017; Gagnaire *et al.* 2012; Pavey *et al.* 2015; Pujolar *et al.* 2014b; Williams *et al.* 1973), but this is expected to be a single generation effect as individuals may end up in environments that differ considerably from those of their parents (Gagnaire *et al.* 2012; Pujolar *et al.* 2014b). The resulting absence of genetically based local adaptation suggests that phenotypic plasticity could play an important role in the species' ability to persist in different environments, although there is also evidence for a role of weak, polygenic selection to occur (Côté *et al.* 2014; Pavey *et al.* 2015). For instance, American glass eels sampled at geographically and environmentally different localities showed different growth rates and transcriptomic reaction norms in common garden settings when exposed to different salinities (Côté *et al.* 2014; Côté *et al.* 2009). This suggests that phenotypic plasticity interacts with processes that have occurred at local scales at a very early stage coinciding with the arrival of glass eels at the sites; either genetic variation shaped by within-generation selection (as the species is panmictic) and/or epigenetic imprints. However, knowledge about differences in methylation across geography and environments is currently lacking.

Finally, European and American eel can hybridize (Albert *et al.* 2006; Avise *et al.* 1990; Jacobsen *et al.* 2017; Pujolar *et al.* 2014a), as is also the case for other Anguillid species (Barth *et al.* 2020). Genomic analyses suggest a protracted speciation process by involving

For Review Only

160 episodes of isolation and secondary contact and with ongoing gene flow (Nikolic *et al.* 2020).
161 Given the incomplete speciation process it is of interest to assess if TE derepression occurs in
162 hybrids between European and American eel.
163
164 Here, we applied both whole genome sequencing and bisulphite sequencing of eels from
165 geographical locations ranging from Iceland to Morocco. As the functional significance of
166 methylation depends on the specific genomic categories (e.g. promotors and exons) being
167 methylated, we first made use of whole genome information to characterize the general
168 methylation landscape of European eel. Subsequently, we 1) tested the hypothesis that
169 differences in methylation is present among glass eels from different localities and are
170 furthermore associated with differences in environmental parameters. Given the panmictic
171 nature of the species we further predicted that such methylation differences should vastly
172 exceed genetic differences, even if within-generation selection occurs. 2) We assessed if
173 methylation differences are present between glass and adult eels, and if so if this can be
174 ascribed to the pronounced stages of metamorphosis or to mere ageing effects (Horvath &
175 Raj 2018). 3) By analysing methylation in European x American eel hybrids we tested the
176 hypothesis that transposon methylation does not differ from pure European eel, reflecting the
177 prolonged and incomplete speciation process.
178
179 **Materials and Methods**
180
181 Samples
182 A total of 50 European eels were analyzed, representing seven locations in Europe and
183 Northwestern Africa, spanning 30 degrees of latitude (Fig. 1, Table 1, Supporting
184 Information, Table S1). The samples were collected between 2001 and 2016 (Table 1).
185 Icelandic samples were collected for the present study, whereas the remaining samples have
186 previously been analyzed using RAD sequencing (Pujolar *et al.* 2014b; Pujolar *et al.* 2015).
187 Thirty-nine individuals were glass eels (juvenile eels recently arrived at the coasts), whereas
188 11 adult individuals (silver eels, i.e. about to undertake their spawning migration) were
189 included from two locations (Burrishoole, Ireland; Valencia, Spain). Three individuals (two
190 from Iceland and one from Ireland) were detected as hybrids with American eel (one F1
191 hybrid and two backcrosses in the direction of European eel), determined using species-
192 diagnostic SNPs (Pujolar *et al.* 2014a). For silver eels, tissues consisted of muscle, whereas
193 for glass eels DNA was extracted from the tail end, composed primarily of muscle. DNA was
194 extracted using the E.Z.N.A.® Tissue DNA Kit (OMEGA, Bio-tek, CA, USA) following the
195 manufacturer's recommendations. Whole-genome sequencing (WGS) and whole-genome-
196 bisulfite sequencing (WGBS) was outsourced to Novogene Europe (Cambridge, UK).
197 Sequencing was conducted 150 bp paired-end on the Illumina HiSeq platform and aimed for
198 a minimum coverage of 10X.
199
200 Mapping WGS reads and calling SNPs
201 The WGS reads were filtered using Trim Galore v0.4.1
202 (https://github.com/FelixKrueger/TrimGalore) and mapped to a recent chromosome level
203 European eel genome assembly (Rhie *et al.* 2021) (GenBank accession: GCA_013347855.1)
204 using the BWA-MEM algorithm of BWA v0.7.17 (Li & Durbin 2009). The resulting SAM
205 files were sorted by coordinate and were converted to BAM format using samtools v1.9 (Li *et*
206 *al.* 2009). A VCF file of SNPs encompassing all 50 individuals was generated from the BAM
207 files using bcftools v1.9 (Li *et al.* 2009), constraining the minimum mapping quality to 20.
208 Only biallelic SNPs with minimum variant quality of 20 and with combined coverage falling

For Review Only

between 500 and 750 were kept. The coverage thresholds were decided upon inspecting the coverage distribution of the SNPs (Supporting Information, Fig. S1).

Genome-wide H_O (observed heterozygosity) of the individuals was calculated from the VCF file by dividing the number of heterozygous sites with adjusted genome lengths. We used this measure to corroborate the hybrid status of individuals and as a quality check of the data; in a panmictic species with expectedly almost no inbreeding, genome-wide H_O should be very similar across individuals. The genome length was adjusted per individual by correcting for the missing sites generated by the SNP calling process. A PCA aimed at analyzing genetic relationships among sampled individuals was conducted using the R function "prcomp" (R Core Team 2018) on the genotype table of the individuals, where the genotypes were denoted as the number of alternative alleles.

Mapping WGBS reads and calling methylation

A total of 36 individuals succeeded in WGBS (Supporting Information, Table S1) with degradation and insufficient yields of DNA causing failure in sequencing of the remaining individuals. The WGBS reads were filtered using Trim Galore by allowing "--trim1" and were mapped to genomes using Bismark v0.22.3 (Krueger & Andrews 2011). Reads were mapped to the individual genomes obtained from the WGS data instead of the general reference genome. This was considered necessary due to the exceptionally high genetic diversity of the European eel (Pujolar *et al.* 2013), leading to lower mapping success when using the reference genome. Default parameters were used except for a relaxed gap penalty ("--rdg 2,1 --rfg 2,1").

We subsequently ran "bismark_methylation_extractor" and "bismark2bedGraph" (Krueger & Andrews 2011) to extract all the sequenced CpG sites together with their methylation status. The information was stored in the COVERAGE files in the output. During the extraction process, the first two base pairs of all the Read 2 files were removed based on the M-bias plots. CpG sites containing mutations were excluded. As CpG is palindromic and complementary CpGs are synchronized in methylation due to dnmt1 activity during DNA replication, complementary CpGs were merged. The COVERAGE files of all the individuals were merged using a custom script. This generated a file where the CpGs of all the individuals were aligned by coordinate. Within each individual, the CpGs with coverage lower than five were marked as missing. CpGs missing in more than half of the individuals were filtered out. CpGs whose combined coverage (across individuals) fell outside the range between 115 and 539 were removed. These coverage thresholds were decided from the coverage distribution (Supporting Information, Fig. S2).

To assess differences in global methylation among individuals, PCA was conducted using the R function "prcomp" on the methylation matrix. The data points were the individuals and the variables were the methylation level of the CpG sites.

Methylation in genomic categories

We used the gene annotation file provided with the reference genome sequence, and for each transcript we defined two potential promoter regions, one is from 1 bp to 500 bp upstream from the TSS (transcription start site), referred to as "promoter_1", and the other from 501 bp to 1000 bp upstream, referred to as "promoter_2. We also identified CpG islands using cpgplot in the EMBOSS v6.6.0.0 package (Madeira *et al.* 2019). We further predicted and annotated the repetitive sequences for the reference genome using RepeatModeler v1.0.11 and RepeatMasker v4.0.9-p2 (http://www.repeatmasker.org). Based on the annotation, we

For Review Only

divided the reference genome of European eel into 17 non-exclusive categories (Supporting Information, Table S2) and examined methylation patterns within each of these categories. The aligned CpGs were assigned into the genomic categories using the "intersect" command of bedtools v2.29.0 (Quinlan & Hall 2010).

Correlation between methylation and gene expression

We anticipated that distinct methylation patterns of the first exons and the promoters of the transcripts would indicate a functional role in regulating gene expression. We furthermore also considered first introns, as Anastasiadi *et al.* (2018) reported inverse relationships between methylation of this genomic category and gene expression. Gene expression profiles were not generated in the present study, but we used a published transcriptome dataset of the European eel (Bracamonte *et al.* (2019b) NCBI BioProject: PRJNA419718 and PRJNA547691) as an approximate measure. This means that we could assess patterns of general association between gene expression and methylation in genomic categories, but not specific changes in gene expression as a result of differential methylation. The dataset is comprised of Illumina paired-end reads from 30 experiments (20 individuals). The reads were filtered using Trim Galore v0.4.1 and were mapped to the reference genome with the guidance of the gene annotation using HISAT2 v2.1.0 (Kim *et al.* 2019). The expression profile of all the transcripts was called for each experiment using StringTie v2.0 (Kovaka *et al.* 2019). Upon inspecting the expression profile and visually examining the transcripts in IGV v2.7.2 (Robinson *et al.* 2011), five experiments were removed due to low numbers of expressed transcripts.

The expression levels (measured using FPKM, Fragments Per Kilobase of transcript per Million mapped reads) of the transcripts were averaged across the experiments, and the average values were used to correlate with the methylation levels of the first exons, first introns and the promoters. The methylation level for each first exon, first intron or promoter was represented with the average methylation level across all the individuals.

Identification of lowly-methylated sites (LMSs)

Due to the importance of lowly-methylated sites (LMSs) in activating genes in an otherwise globally methylated genome (Nakamura *et al.* 2014), we inspected the genome-wide distribution pattern of LMSs. In order to include all meaningful LMSs across all individuals, a CpG with a methylation level lower than 0.05 in at least two individuals was defined as an LMS. This threshold was chosen because the CpGs of this methylation level showed the highest tendency of clustering together (Supporting Information Fig. S3). The criterion of a minimum of two individuals aimed to decrease the possibility of false positives caused by modest sequencing coverage.

Association of SNPs and methylation with localities and environmental parameters

We used redundancy analysis (RDA, (Forester *et al.* 2018; Legendre & Legendre 2012)) to study association of SNPs and methylation, respectively, with local environments. The analysis was conducted in the "vegan" package (v2.5-6 (Oksanen *et al.* 2008)) in R. Only non-hybrid glass eels were included in the analyses, encompassing 32 individuals for the SNP and 25 for the methylation data. Two rounds of RDA were implemented. The first round had sampling locations (dummy variables) as explanatory variables. This analysis aimed to examine the genetic or methylation response to the sampling locations regardless of their environmental composition. The second round had sea surface temperature (SST30), chlorophyll concentration and mean day length (MDL) as explanatory variables (Table 1), all encompassing means of 30 days prior to the date of sampling. This analysis can be regarded

For Review Only

as representing a targeted subset of the (unknown) environmental composition represented by the sampling locations. Remotely sensed sea surface temperatures encompassing a resolution of 0.25 degree latitude × 0.25 degree longitude on a global grid and measured for each day, were provided by the NOAA/OAR/ESRL PSD, Boulder, Colorado, USA, (http://www.esrl.noaa.gov/psd/) and retrieved using the function extractOISSTdaily from the R script NOAA_OISST_ncdf4.R (http://lukemiller.org/index.php/2014/11/extracting-noaa-sea-surface-temperatures-with-ncdf4/). Data on chlorophyll concentration were extracted from the CCI-OC Data Portal (https://www.oceancolour.org/) (Sathyendranath *et al.* 2019). Mean day length data were obtained from the Photoperiod Calculator at https://www.ou.edu/research/electron/internet/solarjav.html. SST30 and chlorophyll concentration were included to reflect basic abiotic and biotic properties of the environments, whereas MDL was included to represent diurnal and seasonal variation among localities, possibly associated with e.g. genetic or methylation variation at circadian genes. By including mean values for the 30 days preceding sampling, we aimed to capture as much as possible the environmental conditions the glass eels were exposed to either at the site or close to the site during the last stages of oceanic transport.

RDA was conducted for the SNP and methylation data separately. For the SNP data, we filtered out loci with overall minor allele count lower than four and removed SNPs containing missing values, thus retaining 18,337,468 SNPs. For the methylation data, we filtered out CpGs with more than five missing values across individuals, retaining 1,934,985 CpGs. Missing values in the methylation data were replaced with cross-individual mean values of the corresponding CpGs. The p-value of each RDA was calculated through 5000 permutations and p-values of the RDA axes were calculated using 2000 permutations each. For each significant RDA axis, we extracted the loadings of the SNPs or the CpGs. SNPs or CpGs with extreme loadings were defined as outliers. For SNPs, we used four times the standard deviation away from the mean as the threshold. For CpGs, we used three times the standard deviation away from the mean as the threshold. This difference in thresholds was used to obtain comparable number of outliers between the two datasets.

<u>Methylation related to developmental stage and hybridity</u>

We used P_{ST} combined with methylation difference to search for methylation functionally related to developmental stage and hybridity. P_{ST} is a measure of phenotypic differentiation between groups (Leinonen *et al.* 2013; Pujol *et al.* 2008), here accommodated to evaluate methylation divergence between groups in developmental stage or hybridity and calculated using a custom script in R. We adopted criteria of P_{ST} higher than 0.8 and methylation difference higher than 0.35 to define outliers. For the developmental stage, we compared adults and glass eels. For each group, each CpG had to be scored for at least two individuals.

The outliers were assigned to the 17 genomic categories defined above to check for enrichment. The outlier enrichment in a category was calculated as:

$$outlier\ enrichment = \log_2 \frac{O(frq)}{E(frq)}$$

where *O(frq)* was the observed frequency of the outliers in the category, and *E(frq)* was the expected frequency by random chance. *E(frq)* was obtained by assigning all the CpGs to the genomic categories. A positive enrichment implies overrepresentation of the outliers in the category, and a negative enrichment indicates underrepresentation. The significance of the enrichment was tested by comparison to the confidence interval of null hypothesis, i.e., no enrichment. The confidence interval was defined using binomial distribution.

For Review Only

358 GO term enrichment analysis for the outliers
359 For each set of outliers (both SNPs and methylation data), we extracted all genes that
360 overlapped with the outliers within a 3000 bp range upstream and downstream. The 3000-bp
361 threshold was decided according to the median length of the intergenic regions (Supporting
362 Information, Table S2, Fig. S4). The resulting gene list was tested for GO (gene ontology)
363 term enrichment using the "weight01" algorithm of the "topGO" package (Alexa *et al.* 2006;
364 Schulz *et al.* 2007) in R. The p-values of the GO terms were adjusted following Benjamini-
365 Hochberg procedure (Benjamini & Hochberg 1995). The GO IDs of the genes were retrieved
366 by blasting the genes against the Swiss-Prot database (Release 2021_03, The UniProt
367 Consortium 2019).
368
369 Defining DMRs from the outliers
370 We developed a method for identifying DMRs (differentially methylated regions) from a set
371 of methylation outliers based on the distribution of the neighboring distances among the
372 outliers. Such distributions show two peaks. The peak with a higher mean is a geometric
373 distribution, representing neighbouring distances of randomly distributed outliers with
374 uncertain functional value and not easily separable from noise. In contrast, the peak with
375 lower mean represents regions with multiple closely located outliers, hence strongly
376 indicative of functional roles. We identified these regions as DMRs and obtained them by
377 grouping the outliers in the small-mean peak according to distance. A threshold, *K*, was set
378 for the minimum number of outliers required in a DMR, in order to filter out the noise
379 generated by the large-mean peak. *K* was obtained from the following inequality.

380 $$\frac{\sum_{X=K}^{\infty} E(a_X)}{\sum_{X=K}^{\infty} O(a_X)} < FDR$$

381
382 Here, a_X represents the number of DMRs containing *X* outliers. FDR is the false discovery
383 rate, which we set as 0.01 in this study. $O(a_X)$ is the observed series of a_X, and $E(a_X)$ is the
384 expected series of a_X. $E(a_X)$ was calculated as:

385 $$E(a_X) = N \cdot p^{X-1} \cdot (1-p)^2$$

386 where *N* is the number of neighboring distances in the large-mean peak assuming a geometric
387 distribution, and *p* is the expected ratio of neighboring distances in the small-mean peak
388 under this distribution.
389
390 **Results**
391
392 Genetic variation
393 An overview of whole genome sequencing and mapping statistics for each individual is
394 provided in Supporting Information, Table S3. A total of 74,040,803 SNPs were obtained
395 from the WGS data. The majority of individuals showed highly similar levels of
396 heterozygosity (Supporting Information Fig. S5), ranging from 0.00981 to 0.01084 (mean:
397 0.01028), whereas the three admixed individuals exhibited higher levels (0.01156 to
398 0.01232). Four individuals from Morocco showed higher heterozygosity (0.01161 to
399 0.01229), almost similar to the hybrids. However, species-diagnostic SNPs (Pujolar *et al.*
400 2014a) confirmed them not to be hybrids. Such variation in heterozygosity would not be
401 expected in a panmictic species with high effective population size, and we found that the
402 elevated heterozygosity was most likely due to cross-sample contamination (see Supporting
403 Information, Note S1), with contamination rate ranging from 3.4% to 6.0%. We therefore
404 excluded these individuals from all subsequent analyses, although we note that we observed
405 no noticeable impacts on the methylation-based analyses. A Principal Components Analysis

For Review Only

(PCA) based on SNPs (Fig. 2.a) showed virtually no divergence between the majority of individuals, consistent with the assumption of panmixia of the species (Als *et al.* 2011; Enbody *et al.* 2021; Palm *et al.* 2009; Pujolar *et al.* 2014b), whereas the three hybrids showed separation along PC1 and PC2.

Global methylation

An overview of whole genome bisulphite sequencing, mapping statistics and methylation for each individual is provided in Supporting Information, Table S4. Methylation was analyzed in 33 individuals (after removal of contaminated individuals). A total of 7,484,974 CpG sites were identified (out of 24,369,391 sites in the reference genome). The overall methylation of 70.9% is within the range observed in vertebrates (Head 2014) and of similar magnitude as observed in fishes like three-spined stickleback (*Gasterosteus aculeatus*) (70.3%) (Metzger & Schulte 2018) and zebrafish (*Danio rerio*) (80%) (Feng *et al.* 2010). The methylation level distribution exhibited a U-shape with the two peaks at the ends of the range of methylation (corresponding to hyper and hypomethylation; Fig. 2.c), and this pattern was highly consistent across individuals. A PCA based on methylation of CpG sites separated adult and glass eels along PC1 and PC2 (Fig. 2.b).

Methylation in genomic categories

We divided the reference genome into 17 non-exclusive functional categories according to genomic annotation (Supporting Information, Table S2) and found methylation in most categories, including first introns to be high (Fig. 3). However, promoters and the first exons were notable exceptions and showed the lowest methylation levels. Visual inspection of the methylation level across the chromosomes also indicated a high frequency of methylation valleys at the beginning of genes (see Supporting Information, Fig. S6 for an example from Chr_01). The methylation level of each CpG was highly consistent across individuals for the promoters and the first exons (Fig. 3).

Comparison of methylation and gene expression using the transcriptome dataset by Bracamonte *et al.* (2019a) showed that genes with hypomethylated first exons were overall highly expressed, and those with hypermethylated first exons generally showed lower expression levels (Fig. 4.a). This contrasted with first introns (Fig. 4.b), where only weak association was found between hypo- and hypermethylation and gene expression. For promoter 1 and 2 regions there was association between hypo- and hypermethylation and gene expression, although the patterns were weaker than for first exons (Fig. 4.c and d).

We also found that repetitive regions, especially transposable elements (TEs), showed very low numbers of hypomethylated CpGs (Supporting Information, Fig. S7), likely related to TE silencing. This was further supported by the observation that the CpGs in TEs exhibited the lowest methylation variation (Fig. 3).

We found that the CpG islands were overall highly methylated (Fig. 3), in accordance with other studies (Deaton & Bird 2011) and in contrast to previous notions that the hypomethylated CpGs are mainly confined to the CpG islands in promoter regions (Saxonov *et al.* 2006). The CpG islands were not enriched in promoters (Supporting Information, Fig. S8), but were enriched in CDS and LTR, both being highly methylated.

PCA performed for each of the genomic categories generally separated adults and glass eels (Supporting Information, Fig. S9), similar to genome-wide methylation patterns (Fig. 2.b).

For Review Only

455 This suggests that the methylation differences between developmental stages exist in all
456 categories and is the dominant source of variance.
457
458 Characteristics of lowly-methylated sites (LMSs)
459 We identified 1,099,209 lowly-methylated CpG sites (LMSs), which tended to cluster into
460 local groups (Supporting Information, Fig. S3). They were highly enriched in the promoters
461 and the first exons and were underrepresented in the repetitive sequences (Supporting
462 Information, Fig. S10). Some genomic regions exhibited particularly high density of LMSs
463 (Supporting Information, Fig. S11). The gene clusters and genes identified in these regions
464 included: all eight hox clusters of the European eel (see Supporting Information Fig. S11 and
465 Table S5 for genomic coordinates), the two largest protocadherin clusters (12 copies on
466 Chr_03 and 6 copies on Chr_09), the two largest olfactory receptor clusters (110 copies on
467 Chr_09 and 125 copies on Chr_12) and a zscan2 cluster (5 copies on Chr_08). The genes
468 included: zic gene pairs, tbx, tfap2 and homeobox genes other than hox. The hox and
469 protocadherin clusters, homeobox genes, zic pairs and tbx have previously been reported in
470 medaka fish (*Oryzias latipes*) and/or threespine stickleback to reside in large hypomethylated
471 domains (Metzger & Schulte 2018; Nakamura *et al.* 2014), hence consistent with their high
472 density of LMSs found in this study. We provide an extended description of the distribution
473 of LMSs in Supporting Information, Note S2.
474
475
476 Genetic and methylation response to local environments
477 Redundancy analysis [RDA (Forester *et al.* 2018; Legendre & Legendre 2012)] was used to
478 study association of genetic or methylation variation with local environments experienced by
479 glass eels. That left 32 individuals for the genetic data and 25 for the methylation data. Two
480 rounds of RDA were implemented, with the first having the sampling locations (dummy
481 variables) as explanatory variables, thus examining the genetic or methylation response to the
482 sampling locations encompassing a range of (undefined) environmental parameters. For the
483 genetic data (SNPs), 0.06% of the variance was explained by the location variables, and for
484 the methylation data (CpG sites), 2.98% was explained (Fig.5a). The results from both
485 datasets were significant (p-value: 0.0028 and 0.0004, respectively). We further extracted
486 outliers from the loading scores of the first axes, since for both datasets only the first axes
487 were significant (p-value: 0.0075 and 0.0170, respectively). For the SNP data, 11949 outliers
488 were obtained. GO term enrichment analysis for the surrounding genes revealed high
489 abundance of genes involved in nervous system development and functioning (Supporting
490 Information, Table S6). We inspected the genome-wide distribution of the outliers and found
491 regions with high outlier density (Fig. 5b). However, the genes in these regions do not show
492 much functional overlap with the major GO terms.
493
494 For the methylation data, 23912 outliers were found. After grouping the outliers into regions
495 according to their neighbouring distances, this led to the identification of 1523 DMRs
496 (Supporting Information, Table S7). Genes in these regions were enriched with functions
497 related to developmental processes (Supporting Information, Table S8). The genome-wide
498 distribution of the outliers displayed multiple high-density regions (Fig. 5b). Genes in these
499 regions exhibited high correlation with the major GO terms. In particular, seven of the eight
500 hox clusters turned out to be high-density regions.
501
502 The second round of RDA had sea surface temperature (SST30), mean day length (MDL) and
503 chlorophyll concentration as explanatory variables, all representing means over the last 30
504 days prior to the date of sampling. Similar to the first round of RDA, less variance (0.03%)

For Review Only

was explained by environmental variables for the genetic data as compared to the methylation data (1.36%; Fig. 6a). The results were significant for both datasets (p-value: 0.0396 and 0.0056, respectively), but only the first axes were significant or marginally significant (p-value: 0.0915 and 0.0325, respectively). A total of 12124 outliers were obtained from the genetic data, with surrounding genes enriched with the same major GO terms as in the RDA involving localities (Supporting Information, Table S9). Similar to the first round of RDA, the genome-wide distribution of the outliers showed some high-density regions (Fig. 6b), but with no functional overlap of major GO terms observed for the genes in these regions. For the methylation data, 19311 outliers were found, from which 803 DMRs were identified (Supporting Information, Table S10). Genes in these DMRs were enriched with functions related to developmental processes (Supporting Information, Table S11), and the outliers showed several high-density regions (Fig. 6b), encompassing among others six of the hox clusters. Inspection of outliers associated with the individual environmental parameters did not reveal obvious differences (Supporting Information, Table S12); the major GO terms were primarily associated with development regardless of the environmental parameter. A total of 9% percent of the SNP outliers (1092 SNPs) and 39.5% (7632 CpGs) of the methylation outliers overlapped with those identified in the first round of RDA, reflecting the fact that the three environmental parameters represent a subset of the total environmental variation among sites.

A total of 30 GO terms overlapped between outlier SNPs and DMRs for the RDA involving locality as explanatory variable (6.9% of DMR GO terms), and 7 GO terms overlapped for SNPs and DMRs identified using environmental variables as explanatory variables (2.3% of DMR GO terms). The overlapping GO terms are highlighted in Supporting Information, Table S6 and S9.

<u>Methylation related to developmental stage and hybridization</u>

For developmental stage, the P_{ST} distribution between glass eels and adults indicated that the two groups were divergent in methylation throughout most of the genome, and the methylation difference distribution showed adults to be overall hypomethylated compared to glass eels (Fig. 7.a). Using criteria of $P_{ST} > 0.8$ and methylation difference > 0.35, we identified 10767 hypomethylated and 3411 hypermethylated outliers in adults relative to glass eels. According to the neighbouring distance distribution of the outliers, hypomethylated outliers seemed to be more randomly distributed across the genome whereas hypermethylated outliers were highly targeted (Fig. 7.b). We examined the enrichment of the outliers in the 17 genomic categories defined above. The hypomethylated outliers were significantly enriched in intergenic regions, and hypermethylated outliers were significantly enriched in the intergenic regions, the promoters and the first introns (Fig. 7.c). From the hypomethylated outliers, 389 DMRs were identified (Supporting Information, Table S13). No significantly enriched GO terms were found from the genes residing in these DMRs (Supporting Information, Table S14). From the hypermethylated outliers, 577 DMRs were found (Supporting Information, Table S13), enriched with transcription regulators and development-related genes (Supporting Information, Table S14).

The genome-wide distribution of the outliers corroborated the neighbouring distance distribution (Fig. 7.d). The outliers hypomethylated in adults were primarily randomly distributed. Hence, despite their abundance being three times higher than hypermethylated outliers, only six high-density regions were identified, encompassing four genes that were all related to development and cell differentiation. In comparison, the hypermethylated outliers encompassed 25 high-density regions. The three highest peaks correspond to two copies of nfix and one pura. They are involved in the initiation of DNA replication and transcription

For Review Only

(Gronostajski 2000). Two copies of nfic and one nfia were also among the high-density regions. They have similar functions as nfix, and together they cover five of the six nuclear factor I genes in the European eel genome. Interestingly, the two copies of nfix and nfic were found in both hypomethylated and hypermethylated DMRs (Supporting Information, Table S13). This may be related to alternative splicing of these genes, as the isoforms of these genes tend to have different or even opposite effects (Gronostajski 2000). The genome contains two copies of zic1/zic4 gene pair, and both copies were found within the high-density regions. These genes are crucial for nervous system development in embryos. Three copies of igf2bp genes were found, and there are in total five copies in the genome. However, the hypermethylated DMRs represented all five copies (Supporting Information, Table S13). Igf2bp genes are RNA-binding factors. They play direct roles in transport and translation of mRNAs and protect them from endonuclease and miRNA attacks. Igf2bp plays important role in nervous system development. Two copies of bmi1 were found in the high-density regions, out of the three copies in the genome. They are components of PRC1 complex, which induce gene repression through histone modification. PRC1 regulate many genes during development, including the hox clusters. For the rest of the genes in the high-density regions, most are related to developmental processes. We also note that in contrast to outliers associated with local environments, none of the hox clusters were found in high-density regions, and among DMRs only hoxDa on Chr_03 was visibly hypermethylated (Supporting Information, Table S13).

All adult samples were from Bur and Val, with each locality represented by three adults and three glass eels. We repeated the analysis above for each locality in order to assess parallelism in methylation differences between adults and glass eels. For Bur, 37727 outliers were found, and 6007 of them overlapped with the outliers found above (42.37%; the percentages are relative to the number from the analysis encompassing all adult versus all glass eels). For Val, 36739 outliers were found of which 5411 (38.16%) overlapped. A total of 1860 (13.12%) outliers were shared by all three sets. The percentages remained similar when only hypomethylated or hypermethylated outliers were considered. Under a null hypothesis that the three sets of outliers are uncorrelated, the expected percentages would be 1.95%, 1.90%, 0.037%.

For hybridity, we defined hybrids and the non-hybrids as two groups. The methylation profiles of the two groups based on P_{ST} were only mildly divergent (Fig. 8.a). We obtained 8577 hypomethylated and 667 hypermethylated outliers in hybrids relative to non-hybrids. Both sets of outliers were largely randomly distributed in the genome (Fig. 8.b). The hypomethylated outliers were significantly enriched in intergenic regions, repetitive regions and DNA TEs (Fig. 8.c), with patterns in the last category suggesting demethylation of transposons in hybrids. The hypermethylated outliers were significantly enriched in intergenic regions and promoters (Fig. 8.c). A total of 129 hypomethylated and 90 hypermethylated DMRs were found (Fig. 8.d, Supporting Information, Table S15), with no significant enrichment of any GO terms found for either set of DMRs (Supporting Information, Table S16).

Discussion

The results of this study, along with other recent papers (Christensen *et al.* 2021; Leitwein *et al.* 2021; Wellband *et al.* 2021), represent some of the first data on methylation at the whole genome level in fishes, and we observed a complex methylation landscape that is associated with the general functional roles of methylation. In relation to our specific research objectives

For Review Only

we found 1) outlier SNPs in glass eels with respect to geographical location and environmental parameters, possibly reflecting within-generation selection. 2) In parallel with outlier SNPs we found differentially methylated regions in glass eels associated with geographical locations and local environments, indicating a role in local adaptive responses. Neighbouring genes particularly represented functions related to development, and especially Hox genes were prominent. 3) Life stage (glass and adult eels) was the overall strongest determinant of methylation differences among individuals, and a considerable portion of methylation differences was associated with genes of importance to developmental processes. 4) TEs were highly represented among methylation outliers between hybrids and non-hybrid European eel, and were hypomethylated in hybrids, indicating that TE derepression also occurs in this system of incomplete speciation. We discuss these findings in more detail in the following.

Methylation landscape in European eel

The general methylation landscape was in accordance with findings in other vertebrates (Brenet *et al.* 2011; Feng *et al.* 2010; Head 2014; Metzger & Schulte 2018), including overall high levels of genome-wide methylation but with promotors and first exons showing distinctly lower methylation and also higher variance in methylation. Moreover, the association between gene expression and methylation at first exons and promoters suggested a functional role of methylation in these genomic categories (Brenet *et al.* 2011; Jones 2012). We found only weak association between methylation at first introns and gene expression, hence somewhat contrasting with the results by Anastasiadi *et al.* (2018). On the other hand, however, first introns showed enrichment among hypermethylated outliers in adult as compared to glass eels, suggesting a functional role of this genomic category. Some notable patterns were found in the analysis of lowly methylated sites (LMS), where specific gene clusters and genes, particularly hox, protocadherin and olfactory receptor clusters coincided with large lowly methylated domains, in parallel to findings in medaka and three-spine stickleback (Metzger & Schulte 2018; Nakamura *et al.* 2014). It has been previously suggested that such large hypomethylated domains act to suppress transcription of genes while at the same time retaining flexibility for transcription during development (Nakamura *et al.* 2014). This illustrates the complexity of patterns of methylation, as hypomethylation of promoter regions *per se* is otherwise positively associated with increased transcription (Moore *et al.* 2013).

Genetic and methylation response to local environments

The unique life history of European eel causes it at the same time to be panmictic and yet to be distributed across geographically and environmentally highly divergent localities (Als *et al.* 2011; Enbody *et al.* 2021; Pujolar *et al.* 2014b), hence providing opportunities to assess epigenomic responses to environmental variables independent of population-specific genetic variation. However, despite panmixia, redundancy analyses nevertheless showed a low but significant proportion of genetic variance at the level of SNPs that was explained by locality and environmental variables (0.06 and 0.03%, respectively), and > 10,000 outlier SNPs were identified. The finding of genomic regions with high densities of outlier SNPs and enrichment of GO-terms associated with nervous system development and functioning further lends credibility to these findings representing genuine biological signals rather than false positives. In that sense, the findings are in accordance with previous findings in both European and American eel (Babin *et al.* 2017; Gagnaire *et al.* 2012; Pavey *et al.* 2015; Pujolar *et al.* 2014b; Williams *et al.* 1973), ascribed to within-generation selection and involving polygenic selection. The finding of specific genomic outlier regions as opposed to more even distribution of outliers is puzzling, as this would not be likely to occur as a result

For Review Only

of selection within a single generation; panmixia would imply that offspring may end up in very different geographic localities as compared to their parents. Pavey *et al.* (2015) found evidence for polygenic divergence between freshwater and brackish/saltwater ecotypes of American eel and suggested genotype-dependent habitat choice as one possible explanation. It is possible that genotype-dependent habitat choice could also explain our results, but addressing this issue would require other studies and sampling designs.

A significant proportion of the variance in methylation was explained by locality and environmental parameters (2.96 and 1.36%, respectively). The fact that the specific environmental variables mean day length, sea surface temperature and chlorophyll concentration accounted for considerably less of the variance, as compared to locality, suggests that other environmental factors that varies across localities (and/or across years) may have important effects on methylation. One such factor could be local population density; for instance, sex determination in Anguillid eels is principally or exclusively environmentally determined (Geffroy & Bardonnet 2016). It has been suggested that high population density of glass eels leads to predominance of initially fast-growing males and low density predominance of initially slow-growing females (Davey & Jellyman 2005), although the mechanisms and factors involved may be considerably more complex (Côté *et al.* 2015; Geffroy & Bardonnet 2016). Although specific information about density of glass eels at the sampling localities was not available, recruitment is known to vary considerably across the distributional range of the species (Bornarel *et al.* 2018; Dekker 2003; Tesch 2003), with the Bay of Biscay and the Iberian Coast (here represented by the Gironde sample) accounting for a major part of total recruitment (Bornarel *et al.* 2018). Hence, we find it plausible that methylation differences across samples could at least partly reflect differences in population density, also considering the high representation of developmental processes among significant GO-terms for DMRs. Indeed, it has previously been found in a bird species that changes in a social environment leading to increased competition also led to altered patterns of methylation (Rodriguez-Martinez & Galvan 2019).

The finding of DMRs associated with Chlorophyll a concentration and sea surface temperature is not surprising, given the biological importance of these factors. The former of these can be considered a proxy of productivity, thereby affecting feeding and growth of glass eels. It is well established in humans and other vertebrates that diet can affect methylation (Lea *et al.* 2016; Zhang & Kutateladze 2018). It is therefore biologically meaningful that most DMRs were associated with GO-terms related to growth and developmental processes.

The life cycle and behaviour of European eel, including feeding and locomotory activity, is strongly affected by light regimes (Lopez-Olmeda *et al.* 2012; Tesch 2003). Given the variation in mean day length across sampling sites and dates, we anticipated a certain representation of circadian-related genes in DMRs associated with this environmental parameter. In fact, a previous population genomics study found a significant correlation between latitude and the circadian clock gene period (*Per*) indicative of within-generation selection (Pujolar *et al.* 2014b). At the methylation level in the present study, however, no DMRs were associated with circadian genes, although we note that this could be a result of analyzing muscle tissue, whereas circadian genes would be expected to be functionally most important in brain tissue (Baras *et al.* 1998). Similar to the other environmental parameters, significant GO-terms were instead dominated by developmental processes. It is possible that different activity schemes associated with different light regimes could influence

For Review Only

development, and mean day length could also be correlated with other environmental factors affecting development.

Remarkably, hox clusters featured prominently among high-density regions of outliers, both in relation to localities and specific environmental parameters. Hence, hox clusters are not only generally situated in large hypomethylated domains, but also represent some of the genomic regions showing most pronounced differential methylation among individuals from different localities and environments. Hox genes are of fundamental importance in developmental processes, notably with respect to determining body plans (Carroll 2008; Duboule 2007). It has previously been found that eels have retained a surprisingly large repertoire of duplicated hox clusters and this has been suggested to underlie the two different body plans of the leaf-shaped larval stage (leptocephalus) and the glass and adult eel stages (Henkel *et al.* 2012). Our results suggest that variation in methylation of hox genes (and by inference their regulation) could also be associated with phenotypic variation that develops in response to local environmental conditions. Our data do not allow for specifically associating methylation with phenotypic traits. However, examples of morphological variation exist in adult eels such as distinct narrow-headed and broad-headed types that exhibit different feeding preferences; these morphs are associated with different transcriptomic profiles already at the glass eel stage (De Meyer *et al.* 2017).

Other explanations should, however, also be considered, as glass eels arriving at different localities could show different ages and development, for instance involving different methylation of hox clusters. Hence, if recruitment of glass eels exclusively occurs via the Gulf Stream, then glass eels would be expected to be younger in northern as opposed to southern localities. However, other ocean currents than the Gulf Stream are assumed to be involved in transport of larvae (Munk *et al.* 2010), age determination of glass eels is generally considered controversial (Bonhommeau *et al.* 2010), and it has been suggested that distance from inshore regions to the Continental Shelf could be the primary factor affecting age of newly recruited glass eel (Lecomte-Finiger 1992). Hence, this scenario merits consideration, but is not possible to assess with the data and knowledge of recruitment patterns at hand.

Our results show that already at the early life stage of glass eel, where individuals settle in their future nursery and foraging areas, differential methylation is present that is associated with geographical locations and/or environmental parameters. These differences have the potential to affect gene expression and phenotypes also later in life, and the results raise the possibility that epigenetics could in fact underlie differences in growth rates and transcriptomic reaction norms as observed in American eels from different localities (Côté *et al.* 2014; Côté *et al.* 2009). Nevertheless, it is a complex question if the methylation differences lead to phenotypic plasticity of adaptive value. This would require that environmental factors affecting methylation should also be predictive of the environmental conditions encountered later in life (Bateson *et al.* 2014). Analysis of methylation in older (yellow) eels from different localities and environments could shed further light on the role of methylation in adaptive processes in eels, and if the same cohorts could be followed from the glass eel stage this could allow for assessing the temporal stability and adaptive significance of methylation differences induced in early life. Finally, although our focus on a panmictic species should minimize interactions between genetic variation and methylation, the results also show some genetic variation associated with environmental factors. A genetic influence on methylation patterns can therefore not be ruled out entirely, although we note that the functional overlap between outlier SNPs and DMRs was limited. Richards (2006)

For Review Only

distinguished between different categories of epigenetic variation, where genetic variation controls (*obligatory*) or influences (*facilitated*) epigenetic variation, whereas in the *pure* category epigenetic variation is independent of genetic variation. Indeed, empirical evidence now exists from a range of organisms showing that at least a part of methylation variation interacts with or is controlled by genetic variation (i.e. *obligatory* or *pure* epigenetic variation) that may furthermore interact with environmental conditions (Berbel-Filho *et al.* 2019; Dubin *et al.* 2015; Teh *et al.* 2014). It would be an interesting future research question if a higher proportion of epigenetic variation associated with environmental factors is *pure* in panmictic eels as compared to other species showing genetic differentiation and local adaptation across populations. However, this would obviously require a deeper understanding of interactions at the genetic and epigenetic levels, along with comparable data from relevant species.

Differences in methylation between life stages

Patterns of global methylation clearly separated juveniles (glass eels) from adults (silver eels). Samples from juvenile eels could encompass other tissues than muscle, but muscle would nevertheless constitute the bulk of tissue analyzed. We therefore find it less likely that the patterns of methylation should reflect different tissues as opposed to different life stages. Hence, with this caveat in mind life stage was the most important source of inter-individual variation in methylation, and it is noteworthy that this pattern showed high parallelism across the two environments from which adult eels were sampled. The European eel life cycle is characterized by several metamorphoses; from larvae to glass eel, from glass eel to yellow (adult) eel, and from yellow eel to mature silver eel, all involving distinct morphological and physiological changes (Tesch 2003). The extensive methylation differences observed could reflect extensive change of methylation associated with metamorphosis, as previously found in both vertebrate and invertebrate species (Covelo-Soto *et al.* 2015; Gegner *et al.* 2021; Kyono *et al.* 2020), but could also represent more gradual age-related changes in methylation (Horvath 2013; Horvath & Raj 2018; Issa 2014). In that sense it was interesting that outliers that were hypomethylated in adult eels showed a relatively random genomic distribution, whereas hypermethylated regions showed a more targeted genomic distribution with enrichment of promoter regions and first introns. Genome-wide hypomethylation and hypermethylation of promoters is in fact a general pattern of methylation associated with ageing (Johnson *et al.* 2012).

On the other hand, the strong representation of developmental processes among GO terms for hyper-methylated outliers supports links to metamorphic processes. Moreover, whereas our study analyzed methylation in muscle tissue, a previous study of European eel using methylation-sensitive amplified polymorphisms (MSAP) and comparing life stages found little divergence in liver tissue but larger differences in gill and brain tissues (Trautner *et al.* 2017). These tissue-specific differences argue against merely age-related effects and support methylation differences being due to specific traits and environmental conditions encountered by the life stages, e.g. fresh or brackish water in yellow eels and oceanic salinities to be encountered during the spawning migration of silver eels. In the case of muscle tissue, important differences in metabolic capacity and power output also develop between the yellow and silver eel stages (Egginton 1986; Ellerby *et al.* 2001), ascribed to their long spawning migration. However, since our sampling included glass and silver eels, but not yellow eels, it remains uncertain exactly at which life stages the observed methylation differences have occurred. In total, it is possible that the distinct differences in methylation between glass and silver eels could reflect both ageing and metamorphosis, and it would require more extensive analysis of individuals at different age stages to fully resolve this.

For Review Only

Interestingly, whereas hox clusters represented some of the most distinctive methylation outlier regions between glass eels from different localities and environments, they were not represented among methylation outlier regions between juvenile and adult eels, despite their importance in developmental processes. We do not rule out that differential methylation could exist between earlier life stages, notably leptocephali (larvae) and glass eels (as implicitly suggested by Henkel *et al.* (2012)). However, for the life stages covered in this study, differential methylation of hox genes appears almost exclusively associated with environments. This decreases the possibility that the results obtained from glass eels could represent artefacts such as subtle differences in ages and developmental stages among individuals from different localities, as discussed previously.

Methylation in European x American eel hybrids

Transposable elements (TEs) can be considered genomic parasites, and free proliferation of TEs in the genome is harmful. Hence, TEs are inactivated in particular by methylation mediated by small piRNA interacting with PIWI proteins (Goodier 2016). Evolutionary "arms races" between TEs and genes in the PIWI-piRNA pathway has led to rapid evolution and divergence between species, that again results in incompatibilities in hybrids leading to derepression of TEs (Aravin *et al.* 2007; Simkin *et al.* 2013). The resulting remobilization of TEs has been suggested as an important postzygotic reproductive isolation mechanism, even in cases of recent speciation (Laporte *et al.* 2019; Michalak 2009; O'Neill *et al.* 1998; Ungerer *et al.* 2006).

The speciation history of European and American eel is complex and prolonged involving an initial period of reproductive isolation, presumably due to vicariance, followed by secondary contact and ongoing gene flow (Nikolic *et al.* 2020). Genomic outlier regions separating the species primarily represent genes related to energy and development, consistent with differences in length of spawning migration and larval phase duration of the two species (Jacobsen *et al.* 2014). Our results suggest, however, that postzygotic isolation does not only involve selection at ecologically important genes, but could also encompass intrinsic incompatibilities leading to demethylation of TEs, even despite ongoing gene flow. Even though we reject the hypothesis that transposon methylation does not differ between pure European eel and hybrids, we stress that the results presented here are preliminary and do not involve a comparison with the epigenome of pure American eel. Analysis of higher numbers of F1 hybrids and backcrosses could shed further light on TE demethylation and the extent to which it decays with each generation of backcrossing.

Conclusions

Our study of a panmictic species where genetically based local adaptation cannot occur yielded important insights into the much debated issue of the ecological and adaptive role of methylation (Bossdorf *et al.* 2008; Flores *et al.* 2013; Rey *et al.* 2020; Verhoeven *et al.* 2016). In the absence of genetic differentiation and at most a limited degree of within-generation selection, variance in methylation between life stages, between hybrids and non-hybrids and between glass eels from different localities and environments was pronounced. Whereas the variance associated with life stages and hybridization concerns innate properties of the species, the association of methylation with localities and environmental variables does suggest that the genomes of eels can respond epigenetically to local conditions. We cannot entirely rule out the possibility that the genetic variation found to be associated with local environments could also interact with methylation. It can therefore not be concluded directly that methylation "substitutes" genetically based local adaptation, and this would also require

For Review Only

demonstration of the phenotypic effects of methylation and its adaptive value. However, there is certainly the possibility that at least some environmentally induced methylation at the glass eel stage is of adaptive value later in life. There are as yet few comparable studies of wild species quantifying variation of methylation in response to environmental factors. However, the 2-3% of methylation variation associated with localities and environments in the early life stage of glass eel is considerably higher than the ca. 0.01% of methylation associated with different salinities in experiments with three-spined sticklebacks (Metzger & Schulte 2018), but lower than the ca. 16% associated with river and hatchery environments in Coho salmon (*Oncorhynchus kisutch*) (Le Luyer *et al.* 2017).

In total, our study of a panmictic species shows that despite no genetic differentiation a portion of epigenetic variation is associated with local conditions and may contribute to adaptation of individuals. Along with other studies focusing on asexual species or species almost devoid of genetic variation (Angers *et al.* 2010; Berbel-Filho *et al.* 2019; Leung *et al.* 2016; Liu *et al.* 2019; Verhoeven & Preite 2014), or analyzing methylation-environment association while controlling for genetic structure (Gugger *et al.* 2016; Wogan *et al.* 2020), this provides evidence for the biological significance of epigenetic variation while controlling for aspects of genetic variation.

For Review Only

Acknowledgements

We thank Annie Brandstrup for technical assistance, Russel Poole, Eric Feunteun, Françoise Daverat, Gregory Maes and Håkan Wickström for providing samples and two anonymous reviewers for constructive comments and suggestions. This work was funded by The Danish Council for Independent Research, Natural Science (Grant No. 7014-00167B to MMH) and MarGen II, an Interreg project under the Øresund-Kattegat-Skagerrak program.

Author contributions

MMH, SL and LB conceived and designed the study, SL conducted bioinformatics and statistical analyses, ANBT and MWJ identified and validated hybrid individuals, MP, BJ and JLC provided samples and information, SL and MMH wrote the manuscript with input from all other authors. All authors read and approved the final version of the manuscript.

Data Accessibility and Benefit Sharing

Raw data files for both WGS and WGBS are available at NCBI (The National Center for Biotechnology Information) with accession number PRJNA812038. Data files with SNPs (VCF) and methylated sites and scripts used for analyzing the data are available through DRYAD (https://doi.org/10.5061/dryad.q2bvq83nm) (Liu *et al.* 2022).

References

Adrian-Kalchhauser I, Sultan SE, Shama LNS*, et al.* (2020) Understanding 'Non-genetic' Inheritance: Insights from Molecular-Evolutionary Crosstalk. *Trends in Ecology & Evolution* **35**, 1078-1089.

Albert V, Jónsson B, Bernatchez L (2006) Natural hybrids in Atlantic eels (*Anguilla anguilla*, *A. rostrata*): evidence for successful reproduction and fluctuating abundance in space and time. *Molecular Ecology* **15**, 1903-1916.

Alexa A, Rahnenfuhrer J, Lengauer T (2006) Improved scoring of functional groups from gene expression data by decorrelating GO graph structure. *Bioinformatics* **22**, 1600-1607.

Als TD, Hansen MM, Maes GE*, et al.* (2011) All roads lead to home: panmixia of European eel in the Sargasso Sea. *Molecular Ecology* **20**, 1333-1346.

Anastasiadi D, Esteve-Codina A, Piferrer F (2018) Consistent inverse correlation between DNA methylation of the first intron and gene expression across tissues and species. *Epigenetics & Chromatin* **11**.

Anastasiadi D, Piferrer F (2020) A clockwork fish: Age prediction using DNA methylation-based biomarkers in the European seabass. *Molecular Ecology Resources* **20**, 387-397.

Anastasiadi D, Venney CJ, Bernatchez L, Wellenreuther M (2021) Epigenetic inheritance and reproductive mode in plants and animals. *Trends Ecol Evol* **36**, 1124-1140.

Angers B, Castonguay E, Massicotte R (2010) Environmentally induced phenotypes and DNA methylation: how to deal with unpredictable conditions until the next generation and after. *Molecular Ecology* **19**, 1283-1295.

Angers B, Perez M, Menicucci T, Leung C (2020) Sources of epigenetic variation and their applications in natural populations. *Evolutionary Applications* **13**, 1262-1278.

Aravin AA, Hannon GJ, Brennecke J (2007) The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race. *Science* **318**, 761-764.

Artemov AV, Mugue NS, Rastorguev SM*, et al.* (2017) Genome-wide DNA methylation profiling reveals epigenetic adaptation of stickleback to marine and freshwater conditions. *Molecular Biology and Evolution* **34**, 2203-2213.

Avise JC, Nelson WS, Arnold J*, et al.* (1990) The evolutionary genetic status of Icelandic eels. *Evolution* **44**, 1254-1262.

Babin C, Gagnaire PA, Pavey SA, Bernatchez L (2017) RAD-seq reveals patterns of additive polygenic variation caused by spatially-varying selection in the American eel (*Anguilla rostrata*). *Genome Biology and Evolution* **9**, 2974-2986.

Baltazar-Soares M, Biastoch A, Harrod C*, et al.* (2014) Recruitment Collapse and Population Structure of the European Eel Shaped by Local Ocean Current Dynamics. *Current Biology* **24**, 104-108.

Baras E, Jeandrain D, Serouge B, Philippart JC (1998) Seasonal variations in time and space utilization by radio-tagged yellow eels *Anguilla anguilla* (L.) in a small stream. *Hydrobiologia* **372**, 187-198.

Barth JMI, Gubili C, Matschiner M*, et al.* (2020) Stable species boundaries despite ten million years of hybridization in tropical eels. *Nature Communications* **11**, 1433.

Bateson P, Gluckman P, Hanson M (2014) The biology of developmental plasticity and the Predictive Adaptive Response hypothesis. *Journal of Physiology-London* **592**, 2357-2368.

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the royal statistical society. Series B (Methodological)* **57**, 289-300.

Berbel-Filho WM, Rodriguez-Barreto D, Berry N, De Leaniz CG, Consuegra S (2019) Contrasting DNA methylation responses of inbred fish lines to different rearing environments. *Epigenetics* **14**, 939-948.

Bonhommeau S, Castonguay M, Rivot E, Sabatie R, Le Pape O (2010) The duration of migration of Atlantic *Anguilla* larvae. *Fish and Fisheries* **11**, 289-306.

For Review Only

949 Bornarel V, Lambert P, Briand C*, et al.* (2018) Modelling the recruitment of European eel
950 (*Anguilla anguilla*) throughout its European range. *Ices Journal of Marine Science* **75**,
951 541-552.
952 Bossdorf O, Richards CL, Pigliucci M (2008) Epigenetics for ecologists. *Ecology Letters* **11**,
953 106-115.
954 Bracamonte SE, Johnston PR, Knopf K, Monaghan MT (2019a) Experimental infection with
955 *Anguillicola crassus* alters immune gene expression in both spleen and head kidney
956 of the European eel (*Anguilla anguilla*). *Marine Genomics* **45**, 28-37.
957 Bracamonte SE, Johnston PR, Monaghan MT, Knopf K (2019b) Gene expression response to
958 a nematode parasite in novel and native eel hosts. *Ecology and Evolution* **9**, 13069-
959 13084.
960 Brenet F, Moh M, Funk P*, et al.* (2011) DNA methylation of the first exon Is tightly linked to
961 transcriptional silencing. *Plos One* **6**, e14524.
962 Carroll SB (2008) Evo-devo and an expanding evolutionary synthesis: A genetic theory of
963 morphological evolution. *Cell* **134**, 25-36.
964 Christensen KA, Le Luyer J, Chan MTT*, et al.* (2021) Assessing the effects of genotype-by-
965 environment interaction on epigenetic, transcriptomic, and phenotypic response in a
966 Pacific salmon. *G3-Genes Genomes Genetics* **11**, jkab021.
967 Côté CL, Castonguay M, Kalujnaia MS, Cramb G, Bernatchez L (2014) In absence of local
968 adaptation, plasticity and spatially varying selection rule: a view from genomic
969 reaction norms in a panmictic species (*Anguilla rostrata*). *Bmc Genomics* **15**, 403.
970 Côté CL, Castonguay M, Verreault G, Bernatchez L (2009) Differential effects of origin and
971 salinity rearing conditions on growth of glass eels of the American eel *Anguilla*
972 *rostrata*: implications for stocking programmes. *Journal of Fish Biology* **74**, 1934-
973 1948.
974 Côté CL, Gagnaire PA, Bourret V*, et al.* (2013) Population genetics of the American eel
975 (*Anguilla rostrata*): FST = 0 and North Atlantic Oscillation effects on demographic
976 fluctuations of a panmictic species. *Molecular Ecology* **22**, 1763-1776.
977 Côté CL, Pavey SA, Stacey JA*, et al.* (2015) Growth, female size, and sex ratio variability in
978 American eel of different origins in both controlled conditions and the wild:
979 implications for stocking programs. *Transactions of the American Fisheries Society*
980 **144**, 246-257.
981 Covelo-Soto L, Saura M, Moran P (2015) Does DNA methylation regulate metamorphosis?
982 The case of the sea lamprey (*Petromyzon marinus*) as an example. *Comparative*
983 *Biochemistry and Physiology B-Biochemistry & Molecular Biology* **185**, 42-46.
984 Davey AJH, Jellyman DJ (2005) Sex determination in freshwater eels and management
985 options for manipulation of sex. *Reviews in Fish Biology and Fisheries* **15**, 37-52.
986 De Meyer J, Maes GE, Dirks RP, Adriaens D (2017) Differential gene expression in narrow-
987 and broad-headed European glass eels (*Anguilla anguilla*) points to a transcriptomic
988 link of head shape dimorphism with growth rate and chemotaxis. *Molecular Ecology*
989 **26**, 3943-3953.
990 Deans C, Maggert KA (2015) What do you mean, “epigenetic"? *Genetics* **199**, 887-896.
991 Deaton AM, Bird A (2011) CpG islands and the regulation of transcription. *Genes &*
992 *Development* **25**, 1010-1022.
993 Dekker W (2003) On the distribution of the European eel (*Anguilla anguilla*) and its fisheries.
994 *Canadian Journal of Fisheries and Aquatic Sciences* **60**, 787-799.

For Review Only

995 Dubin MJ, Zhang P, Meng DZ*, et al.* (2015) DNA methylation in Arabidopsis has a genetic
996 basis and shows evidence of local adaptation. *Elife* **4**.
997 Duboule D (2007) The rise and fall of Hox gene clusters. *Development* **134**, 2549-2560.
998 Dupont C, Armant DR, Brenner CA (2009) Epigenetics: definition, mechanisms and clinical
999 perspective. *Seminars in Reproductive Medicine* **27**, 351-357.
1000 Egginton S (1986) Metamorphosis of the American eel, *Anguilla rostrata* Leseur .1. Changes
1001 in metabolism of skeletal muscle. *Journal of Experimental Zoology* **237**, 173-184.
1002 Ellerby DJ, Spierts ILY, Altringham JD (2001) Slow muscle power output of yellow- and silver-
1003 phase European eels (*Anguilla anguilla* L.): Changes in muscle performance prior to
1004 migration. *Journal of Experimental Biology* **204**, 1369-1379.
1005 Enbody ED, Pettersson ME, Sprehn CG*, et al.* (2021) Ecological adaptation in European eels
1006 is based on phenotypic plasticity. *Proc Natl Acad Sci U S A* **118**, e2022620118.
1007 Feil R, Fraga MF (2012) Epigenetics and the environment: emerging patterns and
1008 implications. *Nature Reviews Genetics* **13**, 97-109.
1009 Feng SH, Cokus SJ, Zhang XY*, et al.* (2010) Conservation and divergence of methylation
1010 patterning in plants and animals. *Proceedings of the National Academy of Sciences of*
1011 *the United States of America* **107**, 8689-8694.
1012 Flores KB, Wolschin F, Amdam GV (2013) The Role of methylation of DNA in environmental
1013 adaptation. *Integrative and Comparative Biology* **53**, 359-372.
1014 Forester BR, Lasky JR, Wagner HH, Urban DL (2018) Comparing methods for detecting
1015 multilocus adaptation with multivariate genotype-environment associations.
1016 *Molecular Ecology* **27**, 2215-2233.
1017 Gagnaire PA, Normandeau E, Côté C, Hansen MM, Bernatchez L (2012) The genetic
1018 consequences of spatially varying selection in the panmictic American eel (*Anguilla*
1019 *rostrata*). *Genetics* **190**, 725-733.
1020 Gapp K, von Ziegler L, Tweedie-Cullen RY, Mansuy IM (2014) Early life epigenetic
1021 programming and transmission of stress-induced traits in mammals. *Bioessays* **36**,
1022 491-502.
1023 Geffroy B, Bardonnet A (2016) Sex differentiation and sex determination in eels:
1024 consequences for management. *Fish and Fisheries* **17**, 375-398.
1025 Gegner J, Vogel H, Billion A, Forster F, Vilcinskas A (2021) Complete metamorphosis in
1026 *Manduca sexta* involves specific changes in DNA methylation patterns. *Frontiers in*
1027 *Ecology and Evolution* **9**.
1028 Gienapp P, Teplitsky C, Alho JS, Mills JA, Merilä J (2008) Climate change and evolution:
1029 disentangling environmental and genetic responses. *Molecular Ecology* **17**, 167-178.
1030 Goodier JL (2016) Restricting retrotransposons: a review. *Mobile DNA* **7**, 16.
1031 Gore AV, Tomins KA, Iben J*, et al.* (2018) An epigenetic mechanism for cavefish eye
1032 degeneration. *Nature Ecology & Evolution* **2**, 1155–1160.
1033 Greenberg MVC, Bourc'his D (2019) The diverse roles of DNA methylation in mammalian
1034 development and disease. *Nature Reviews Molecular Cell Biology* **20**, 590-607.
1035 Gronostajski RM (2000) Roles of the NFI/CTF gene family in transcription and development.
1036 *Gene* **249**, 31-45.
1037 Gugger PF, Fitz-Gibbon S, Pellegrini M, Sork VL (2016) Species-wide patterns of DNA
1038 methylation variation in *Quercus lobata* and their association with climate gradients.
1039 *Molecular Ecology* **25**, 1665-1680.
1040 Head JA (2014) Patterns of DNA Methylation in Animals: An Ecotoxicological Perspective.
1041 *Integrative and Comparative Biology* **54**, 77-86.

For Review Only

1042 Heckwolf MJ, Meyer BS, Hasler R, *et al.* (2020) Two different epigenetic information
1043 channels in wild three-spined sticklebacks are involved in salinity adaptation. *Science*
1044 *Advances* **6**, eaaz1138.
1045 Henkel CV, Burgerhout E, de Wijze DL, *et al.* (2012) Primitive duplicate Hox clusters in the
1046 European eel's genome. *Plos One* **7**, e32231.
1047 Horvath S (2013) DNA methylation age of human tissues and cell types. *Genome Biology* **14**,
1048 3156.
1049 Horvath S, Raj K (2018) DNA methylation-based biomarkers and the epigenetic clock theory
1050 of ageing. *Nat Rev Genet* **19**, 371-384.
1051 Issa JP (2014) Aging and epigenetic drift: a vicious cycle. *Journal of Clinical Investigation* **124**,
1052 24-29.
1053 Jablonka E (2017) The evolutionary implications of epigenetic inheritance. *Interface Focus* **7**,
1054 20160135.
1055 Jablonka E, Raz G (2009) Transgenerational epigenetic inheritance: prevalence, mechanisms,
1056 and implications for the study of heredity and evolution. *Quarterly Review of Biology*
1057 **84**, 131-176.
1058 Jacobsen MW, Pujolar JM, Bernatchez L, *et al.* (2014) Genomic footprints of speciation in
1059 Atlantic eels (*Anguilla anguilla* and *A. rostrata*). *Molecular Ecology* **23**, 4785–4798.
1060 Jacobsen MW, Smedegaard L, Sorensen SR, *et al.* (2017) Assessing pre- and post-zygotic
1061 barriers between North Atlantic eels (*Anguilla anguilla* and *A. rostrata*). *Heredity*
1062 **118**, 266-275.
1063 Johnson AA, Akman K, Calimport SRG, *et al.* (2012) The role of DNA methylation in aging,
1064 rejuvenation, and age-related disease. *Rejuvenation Research* **15**, 483-494.
1065 Jones PA (2012) Functions of DNA methylation: islands, start sites, gene bodies and beyond.
1066 *Nature Reviews Genetics* **13**, 484-492.
1067 Jonsson B, Jonsson N, Hansen MM (2022) Knock-on effects of environmental influences
1068 during embryonic development of ectothermic vertebrates. *The Quarterly Review of*
1069 *Biology* **97**, 95-139.
1070 Kawecki TJ, Ebert D (2004) Conceptual issues in local adaptation. *Ecology Letters* **7**, 1225-
1071 1241.
1072 Kim D, Paggi JM, Park C, Bennett C, Salzberg SL (2019) Graph-based genome alignment and
1073 genotyping with HISAT2 and HISAT-genotype. *Nature Biotechnology* **37**, 907-915.
1074 Kleckner RC, McCleave JD, Wippelhauser GS (1983) Spawning of American eel, *Anguilla*
1075 *rostrata*, relative to thermal fronts in the Sargasso Sea. *Environmental Biology of*
1076 *Fishes* **9**, 289-293.
1077 Kovaka S, Zimin AV, Pertea GM, *et al.* (2019) Transcriptome assembly from long-read RNA-
1078 seq alignments with StringTie2. *Genome Biology* **20**, 278.
1079 Krueger F, Andrews SR (2011) Bismark: a flexible aligner and methylation caller for Bisulfite-
1080 Seq applications. *Bioinformatics* **27**, 1571-1572.
1081 Kyono Y, Raj S, Sifuentes CJ, *et al.* (2020) DNA methylation dynamics underlie metamorphic
1082 gene regulation programs in *Xenopus* tadpole brain. *Developmental Biology* **462**,
1083 180-196.
1084 Laporte M, Le Luyer J, Rougeux C, *et al.* (2019) DNA methylation reprogramming, TE
1085 derepression, and postzygotic isolation of nascent animal species. *Science Advances*
1086 **5**, eaaw1644.
1087 Law JA, Jacobsen SE (2010) Establishing, maintaining and modifying DNA methylation
1088 patterns in plants and animals. *Nature Reviews Genetics* **11**, 204-220.

For Review Only

1089 Le Luyer J, Laporte M, Beacham TD*, et al.* (2017) Parallel epigenetic modifications induced
1090 by hatchery rearing in a Pacific salmon. *Proceedings of the National Academy of*
1091 *Sciences of the United States of America* **114**, 12964-12969.
1092 Lea AJ, Altmann J, Alberts SC, Tung J (2016) Resource base influences genome-wide DNA
1093 methylation levels in wild baboons (*Papio cynocephalus*). *Molecular Ecology* **25**,
1094 1681-1696.
1095 Lecomte-Finiger R (1992) Growth history and age at recruitment of European glass eels
1096 (*Anguilla anguilla*) as revealed by otolith microstructure. *Marine Biology* **114**, 205-
1097 210.
1098 Legendre P, Legendre L (2012) *Numerical ecology*, Third English edition. edn. Elsevier,
1099 Amsterdam.
1100 Leinonen T, McCairns RJS, O'Hara RB, Merila J (2013) Q(ST)-F-ST comparisons: evolutionary
1101 and ecological insights from genomic heterogeneity. *Nature Reviews Genetics* **14**,
1102 179-190.
1103 Leitwein M, Laporte M, Le Luyer J*, et al.* (2021) Epigenomic modifications induced by
1104 hatchery rearing persist in germ line cells of adult salmon after their oceanic
1105 migration. *Evolutionary Applications* **14**, 2402-2413.
1106 Leung C, Breton S, Angers B (2016) Facing environmental predictability with different
1107 sources of epigenetic variation. *Ecology and Evolution* **6**, 5234-5245.
1108 Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler
1109 transform. *Bioinformatics* **25**, 1754-1760.
1110 Li H, Handsaker B, Wysoker A*, et al.* (2009) The Sequence Alignment/Map format and
1111 SAMtools. *Bioinformatics* **25**, 2078-2079.
1112 Lind MI, Spagopoulou F (2018) Evolutionary consequences of epigenetic inheritance.
1113 *Heredity* **121**, 205-209.
1114 Liu S, Tengstedt ANB, Jacobsen MW*, et al.* (2022) Dataset for: Genome-wide methylation in
1115 the panmictic European eel (*Anguilla anguilla*). *DRYAD*,
1116 https://doi.org/10.5061/dryad.q5062bvq5083nm.
1117 Liu SL, Aagaard A, Bechsgaard J, Bilde T (2019) DNA Methylation Patterns in the Social
1118 Spider, *Stegodyphus dumicola*. *Genes* **10**, 137.
1119 Lopez-Olmeda JF, Lopez-Garcia I, Sanchez-Muros MJ*, et al.* (2012) Daily rhythms of digestive
1120 physiology, metabolism and behaviour in the European eel (*Anguilla anguilla*).
1121 *Aquaculture International* **20**, 1085-1096.
1122 Madeira F, Park YM, Lee J*, et al.* (2019) The EMBL-EBI search and sequence analysis tools
1123 APIs in 2019. *Nucleic Acids Research* **47**, W636-W641.
1124 Merondun J, Murray DL, Shafer ABA (2019) Genome-scale sampling suggests cryptic
1125 epigenetic structuring and insular divergence in Canada lynx. *Molecular Ecology* **28**,
1126 3186-3196.
1127 Metzger DCH, Schulte PM (2018) The DNA methylation landscape of stickleback reveals
1128 patterns of sex chromosome evolution and effects of environmental salinity.
1129 *Genome Biology and Evolution* **10**, 775-785.
1130 Michalak P (2009) Epigenetic, transposon and small RNA determinants of hybrid
1131 dysfunctions. *Heredity* **102**, 45-50.
1132 Moore LD, Le T, Fan GP (2013) DNA methylation and its basic function.
1133 *Neuropsychopharmacology* **38**, 23-38.

For Review Only

1134 Munk P, Hansen MM, Maes GE, *et al.* (2010) Oceanic fronts in the Sargasso Sea control the
1135 early life and drift of Atlantic eels. *Proceedings of the Royal Society Series B:*
1136 *Biological Sciences* **277**, 3593-3599.
1137 Nakamura R, Tsukahara T, Qu W, *et al.* (2014) Large hypomethylated domains serve as
1138 strong repressive machinery for key developmental genes in vertebrates.
1139 *Development* **141**, 2568-2580.
1140 Nikolic N, Liu SL, Jacobsen MW, *et al.* (2020) Speciation history of European (*Anguilla*
1141 *anguilla*) and American eel (*A. rostrata*), analysed using genomic data. *Molecular*
1142 *Ecology* **29**, 565-577.
1143 O'Neill RJW, O'Neill MJ, Graves JAM (1998) Undermethylation associated with retroelement
1144 activation and chromosome remodelling in an interspecific mammalian hybrid.
1145 *Nature* **393**, 68-72.
1146 Oksanen J, Kindt R, Legendre P, O'Hara RB (2008) vegan: Community ecology package.
1147 Version 1.7–81, http://cran.r-project.org/.
1148 Palm S, Dannewitz J, Prestegaard T, Wickstrom H (2009) Panmixia in European eel revisited:
1149 no genetic difference between maturing adults from southern and northern Europe.
1150 *Heredity* **103**, 82-89.
1151 Pavey SA, Gaudin J, Normandeau E, *et al.* (2015) RAD sequencing highlights polygenic
1152 discrimination of habitat ecotypes in the panmictic American eel. *Current Biology* **25**,
1153 1666-1671.
1154 Pujol B, Wilson AJ, Ross RIC, Pannell JR (2008) Are Q_{ST}-F_{ST} comparisons for natural
1155 populations meaningful? *Molecular Ecology* **17**, 4782-4785.
1156 Pujolar JM, Jacobsen MW, Als TD, *et al.* (2014a) Assessing patterns of hybridization between
1157 North Atlantic eels using diagnostic single-nucleotide polymorphisms. *Heredity* **112**,
1158 627-637.
1159 Pujolar JM, Jacobsen MW, Als TD, *et al.* (2014b) Genome-wide single-generation signatures
1160 of local selection in the panmictic European eel. *Molecular Ecology* **23**, 2514-2528.
1161 Pujolar JM, Jacobsen MW, Bekkevold D, *et al.* (2015) Signatures of natural selection
1162 between life cycle stages separated by metamorphosis in European eel. *Bmc*
1163 *Genomics* **16**.
1164 Pujolar JM, Jacobsen MW, Frydenberg J, *et al.* (2013) A resource of genome-wide single-
1165 nucleotide polymorphisms generated by RAD tag sequencing in the critically
1166 endangered European eel. *Molecular Ecology Resources* **13**, 706-716.
1167 Quinlan AR, Hall IM (2010) BEDTools: a flexible suite of utilities for comparing genomic
1168 features. *Bioinformatics* **26**, 841-842.
1169 R Core Team (2018) R: A language and environment for statistical computing. R Foundation
1170 for Statistical Computing, Vienna, Austria. http://www.R-project.org/.
1171 Rey O, Eizaguirre C, Angers B, *et al.* (2020) Linking epigenetics and biological conservation:
1172 Towards a conservation epigenetics perspective. *Functional Ecology* **34**, 414-427.
1173 Rhie A, McCarthy SA, Fedrigo O, *et al.* (2021) Towards complete and error-free genome
1174 assemblies of all vertebrate species. *Nature* **592**, 737-746.
1175 Richards EJ (2006) Opinion - Inherited epigenetic variation - revisiting soft inheritance.
1176 *Nature Reviews Genetics* **7**, 395-U392.
1177 Robinson JT, Thorvaldsdottir H, Winckler W, *et al.* (2011) Integrative genomics viewer.
1178 *Nature Biotechnology* **29**, 24-26.

For Review Only

1179 Rodriguez-Martinez S, Galvan I (2019) Exposure to a competitive social environment
1180 activates an epigenetic mechanism that limits pheomelanin synthesis in zebra
1181 finches. *Molecular Ecology* **28**, 3698-3708.
1182 Sathyendranath S, Brewin RJW, Brockmann C*, et al.* (2019) An ocean-colour time series for
1183 use in climate studies: the experience of the ocean-colour climate change initiative
1184 (OC-CCI). *Sensors* **19**, 4285.
1185 Saxonov S, Berg P, Brutlag DL (2006) A genome-wide analysis of CpG dinucleotides in the
1186 human genome distinguishes two distinct classes of promoters. *Proceedings of the*
1187 *National Academy of Sciences of the United States of America* **103**, 1412-1417.
1188 Schmidt J (1923) The breeding places of the eel. *Philosophical Transactions of the Royal*
1189 *Society B: Biological Sciences* **211**, 179-208.
1190 Schmitz RJ, Schultz MD, Urich MA*, et al.* (2013) Patterns of population epigenomic diversity.
1191 *Nature* **495**, 193-198.
1192 Schulz WA, Alexa A, Jung V*, et al.* (2007) Factor interaction analysis for chromosome 8 and
1193 DNA methylation alterations highlights innate immune response suppression and
1194 cytoskeletal changes in prostate cancer. *Molecular Cancer* **6**, 14.
1195 Simkin A, Wong A, Poh YP, Theurkauf WE, Jensen JD (2013) Recurrent and recent selective
1196 sweeps in the piRNA pathway. *Evolution* **67**, 1081-1090.
1197 Skvortsova K, Iovino N, Bogdanovic O (2018) Functions and mechanisms of epigenetic
1198 inheritance in animals. *Nature Reviews Molecular Cell Biology* **19**, 774-790.
1199 Slotkin RK, Martienssen R (2007) Transposable elements and the epigenetic regulation of
1200 the genome. *Nature Reviews Genetics* **8**, 272-285.
1201 Stajic D, Perfeito L, Jansen LET (2019) Epigenetic gene silencing alters the mechanisms and
1202 rate of evolutionary adaptation. *Nature Ecology & Evolution* **3**, 491-498.
1203 Sun D, Layman TS, Jeong H*, et al.* (2021) Genome-wide variation in DNA methylation linked
1204 to developmental stage and chromosomal suppression of recombination in white-
1205 throated sparrows. *Molecular Ecology* **30**, 3453-3467.
1206 Taudt A, Colome-Tatche M, Johannes F (2016) Genetic sources of population epigenomic
1207 variation. *Nature Reviews Genetics* **17**, 319-332.
1208 Teh AL, Pan H, Chen L*, et al.* (2014) The effect of genotype and in utero environment on
1209 interindividual variation in neonate DNA methylomes. *Genome Research* **24**, 1064-
1210 1074.
1211 Tesch F (2003) *The Eel* Blackwell Science Ltd, Oxford.
1212 Trautner JH, Reiser S, Blancke T, Unger K, Wysujack K (2017) Metamorphosis and transition
1213 between developmental stages in European eel (*Anguilla anguilla*, L.) involve
1214 epigenetic changes in DNA methylation patterns. *Comparative Biochemistry and*
1215 *Physiology D-Genomics & Proteomics* **22**, 139-145.
1216 Ungerer MC, Strakosh SC, Zhen Y (2006) Genome expansion in three hybrid sunflower
1217 species is associated with retrotransposon proliferation. *Current Biology* **16**, R872-
1218 R873.
1219 Verhoeven KJF, Preite V (2014) Epigenetic variation in asexually reproducing organisms.
1220 *Evolution* **68**, 644-655.
1221 Verhoeven KJF, Vonholdt BM, Sork VL (2016) Epigenetics in ecology and evolution: what we
1222 know and what we need to know. *Molecular Ecology* **25**, 1631-1638.
1223 Wellband K, Roth D, Linnansaari T, Curry RA, Bernatchez L (2021) Environment-driven
1224 reprogramming of gamete DNA methylation occurs during maturation and is
1225 transmitted intergenerationally in Atlantic Salmon. *G3-Genes Genomes Genetics* **11**.

For Review Only

Williams GC, Koehn RK, Mitton JB (1973) Genetic differentiation without isolation in American eel, *Anguilla rostrata*. *Evolution* **27**, 192-204.

Wogan GOU, Yuan ML, Mahler DL, Wang IJ (2020) Genome-wide epigenetic isolation by environment in a widespread Anolis lizard. *Molecular Ecology* **29**, 40-55.

Zhang Y, Kutateladze TG (2018) Diet and the epigenome. *Nature Communications* **9**, 3375.

For Review Only

Figure legends.

Fig. 1. Map showing the sample localities of the analyzed European eels. The colour coding for the localities applies to all the figures in this paper unless specified otherwise.

Fig. 2. Principal Component Analysis (PCA) based on a) SNPs and b) methylation, respectively, showing the relationships among individuals, with numbers in parentheses indicating the percentage of variance explained by each PC. The colours indicate the sampling localities, as shown in Fig. 1. Squares denote adult eels and circles denote glass (juvenile) eels. c) Distribution of methylation level across CpG sites. The blue lines represent single individuals. The red line represents the average across all individuals.

Fig. 3. Methylation in genomic categories. Each line represents one individual, and the colour indicates sampling location as specified in Fig. 1. a) Average methylation level of all the sequenced CpG sites in each category, and b) b) the standard deviation. c) Inter-individual variation of methylation in genomic categories are shown in the lower panel. Each violin represents the distribution of standard deviation of methylation level calculated across individuals per CpG site. Yellow dots indicate medians.

Fig. 4. Correlation between methylation and gene expression, the latter based on gene expression data from Bracamonte *et al.* (2019a) and Bracamonte *et al.* (2019b). Methylation was divided into three categories, i.e., overall, hypomethylated (methylation level <= 0.2) and hypermethylated (> 0.2). We added a value 1e-7 to the expression level to visualize the unexpressed genes in the logarithmic scale. a) Correlation between exon_1 methylation and gene expression. b) Correlation between intron_1 methylation and gene expression. c) Correlation between promoter_1 methylation and gene expression. d) Correlation between promoter_2 methylation and gene expression.

Fig. 5. a) Redundancy analyses (RDA) of SNPs and methylation (CpGs), respectively, using location as explanatory variable. b) Genomic distribution of RDA outliers for SNPs and methylation (CpGs), respectively. Genes associated with high density regions (peaks) are indicated.

Fig. 6. a) Redundancy analyses (RDA) of SNPs and methylation (CpGs), respectively, using environmental parameters (mean day length [MDL], sea surface temperature [SST30] and Chlorophyll concentration [Chlorophyll]) as explanatory variables. b) Genomic distribution of RDA outliers for SNPs and methylation (CpGs), respectively. Genes associated with high density regions (peaks) are indicated.

Fig. 7. Analysis of methylation differences between life stages. a) Joint distribution of P_{ST} and methylation difference between adults and glass eels. The methylation difference was calculated as the average methylation level of adults minus the average methylation level of glass eels. b) Distribution of neighbouring distance (logarithmically scaled) among CpG outliers related to the developmental stage. The left panel represents outliers hypomethylated in adults, and the right panel outliers hypermethylated in adults. c) Outlier enrichment in genomic categories. Positive values indicate overrepresentation of the outliers in the category and negative values implicate underrepresentation. Asterisks indicate significance (alpha = 0.05). d) Genome-wide distributions of hypo- and hypermethylated outliers, respectively. Genes associated with high density regions (peaks) are indicated.

For Review Only

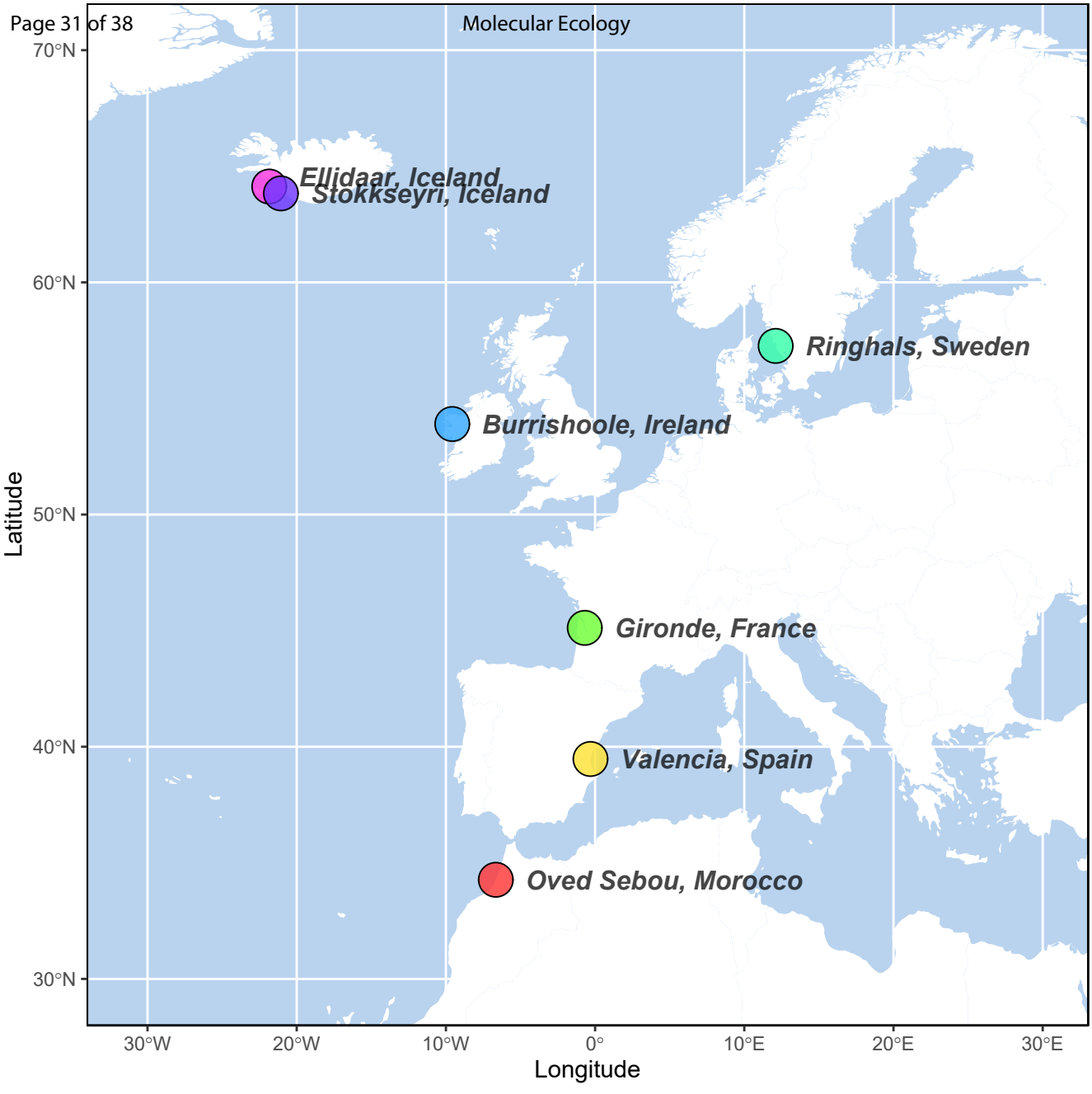
Fig. 8. Analysis of methylation differences between hybrids and non-hybrids. a) Joint distribution of P_{ST} and methylation difference between hybrids and non-hybrids eels. The methylation difference was calculated as the average methylation level of hybrids minus the average methylation level of non-hybrids. b) Distribution of neighbouring distance (logarithmically scaled) among CpG outliers related to hybrid or non-hybrid status. The left panel represents outliers hypomethylated in hybrids, and the right panel outliers hypermethylated in hybrids. c) Outlier enrichment in genomic categories. Positive values indicate overrepresentation of the outliers in the category and negative values implicate underrepresentation. Asterisks indicate significance (alpha = 0.05). d) Genome-wide distributions of hypo- and hypermethylated outliers, respectively. Genes associated with high density regions (peaks) are indicated.

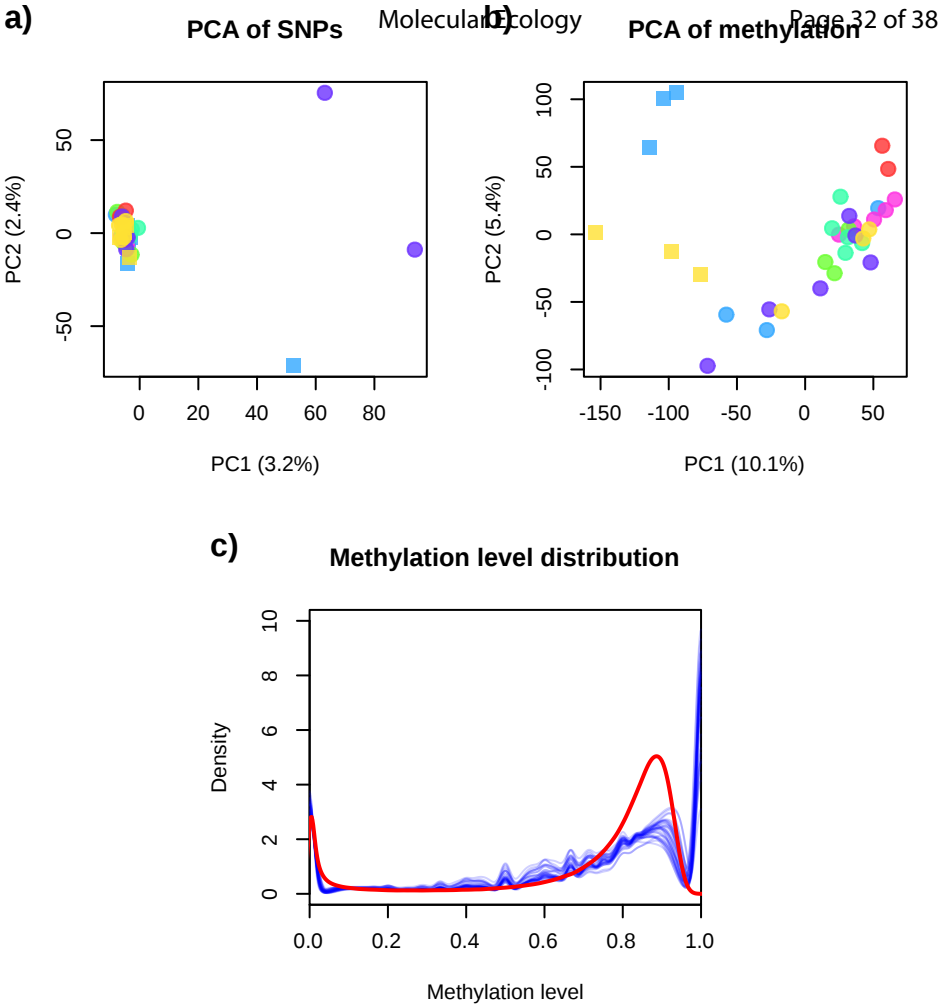
For Review Only

Table 1. Overview of analyzed individuals and environmental parameters. SST30 denotes mean sea surface temperature (degrees C), MDL mean day length (in minutes) and Chlorophyll mean Chlorophyll concentration (mg per m^3) across 30 days prior to sampling. N(WGS) denotes sample size for whole genome sequencing and N(WGBS) sample size for whole genome bisulphite sequencing.

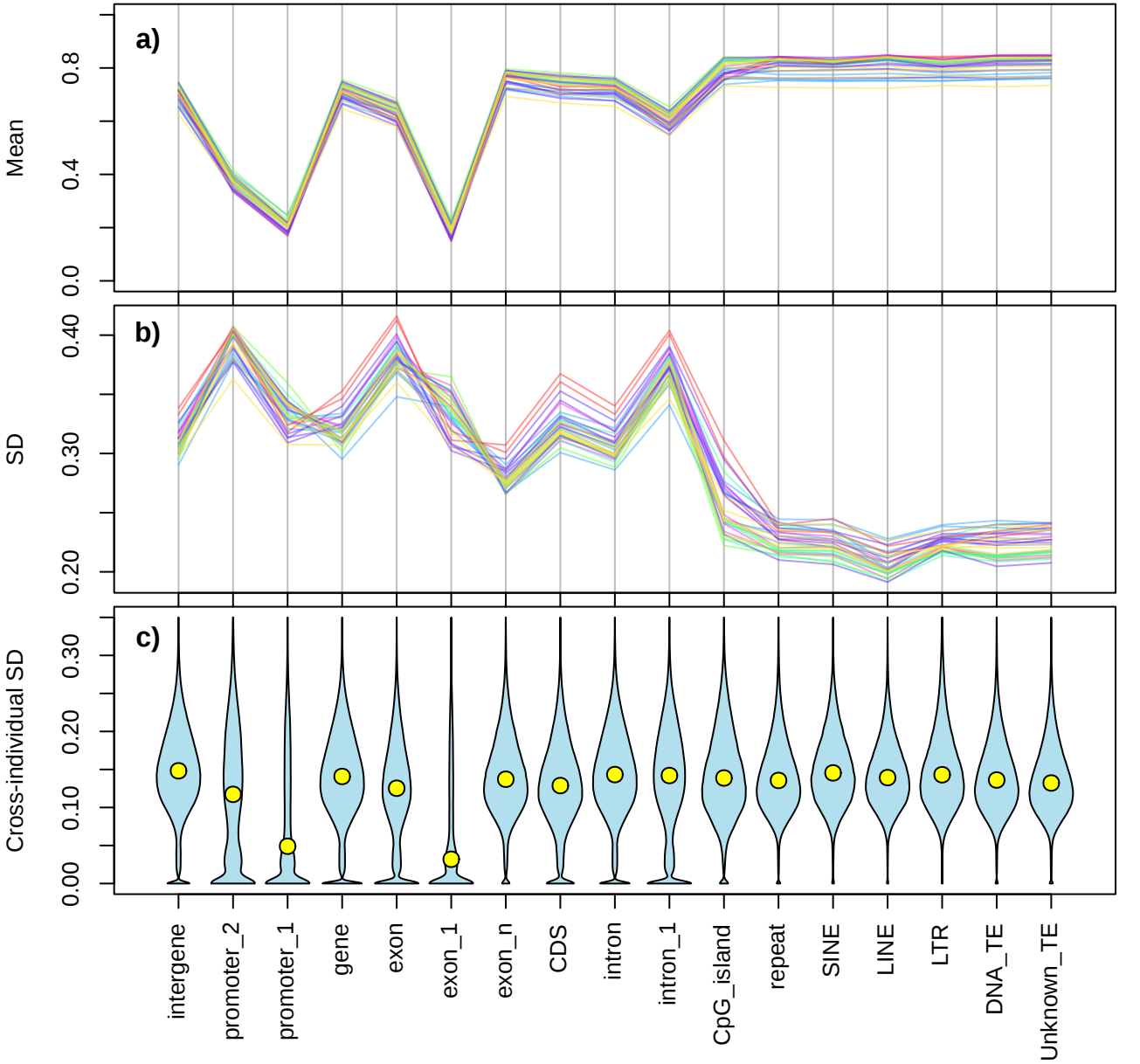
Location	Abbreviation	Time of sampling	Latitude	Longitude	SST30	MDL	Chlorophyll	N(WGS)	N(WGBS)
Glass eels									
Ellidaar, Iceland	Ell	2016-05-16	64.1268	-21.8419	6.26	1015.7	1.371	5	5
Stokkseyri, Iceland	Sto	2016-05-15	63.8324	-21.0603	7.13	1000.2	1.905	6	6
Burrishoole, Ireland	Bur	2008-03-15	53.8989	-9.5742	9.23	640.6	4.48	5	3
Ringhals, Sweden	Rin	2005-03-14	57.2633	12.1025	4.12	633.9	18.175	5	5
Gironde, France	Gir	2008-04-16	45.1193	-0.693	11.33	766.9	11.055	5	3
Valencia, Spain	Val	2010-01-15	39.4724	-0.3107	15.2	562.6	3.17	6	3
Oved Sebou, Morocco	Seb	2001-04-28	34.2698	-6.654	17.5	776.3	2.665	7	5
Adults									
Burrishoole, Ireland	Bur	2010	53.8989	-9.5742	NA	NA	NA	7	3
Valencia, Spain	Val	2012	39.4724	-0.3107	NA	NA	NA	4	3

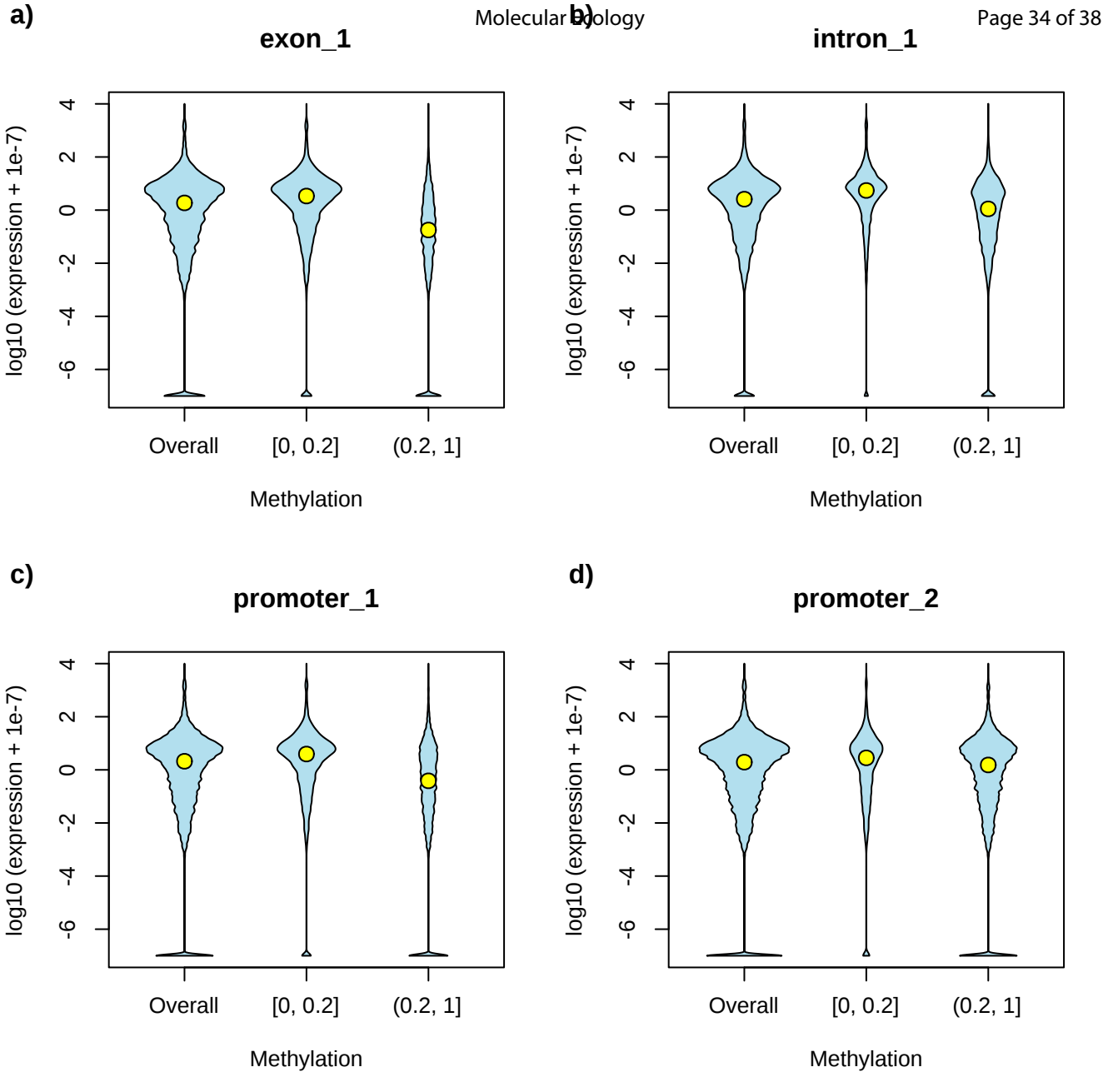
For Review Only


Ellidaar, Iceland
Stokkseyri, Iceland
Ringhals, Sweden
Burrishoole, Ireland
Gironde, France
Valencia, Spain
Oved Sebou, Morocco
70°N
60°N
50°N
40°N
30°N
Latitude
30°W
20°W
10°W
0°
10°E
20°E
30°E
Longitude

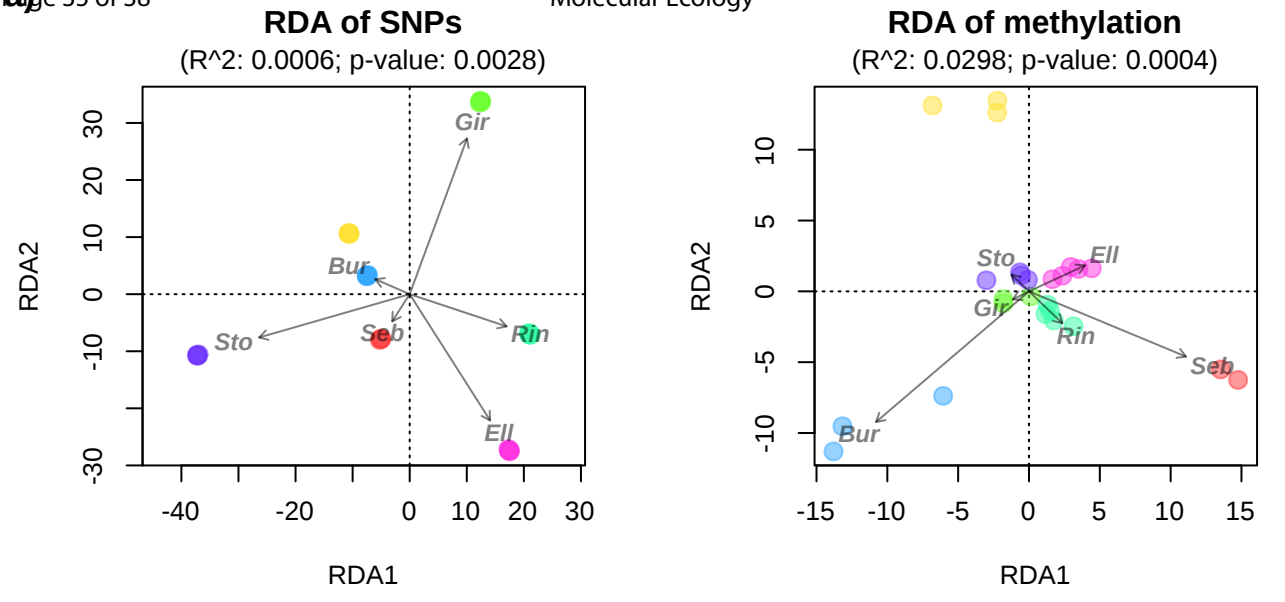

a)
PCA of SNPs
PC2 (2.4%)
PC1 (3.2%)
b)
PCA of methylation
PC2 (5.4%)
PC1 (10.1%)
c)
Methylation level distribution
Density
Methylation level

Methylation of genomic categories

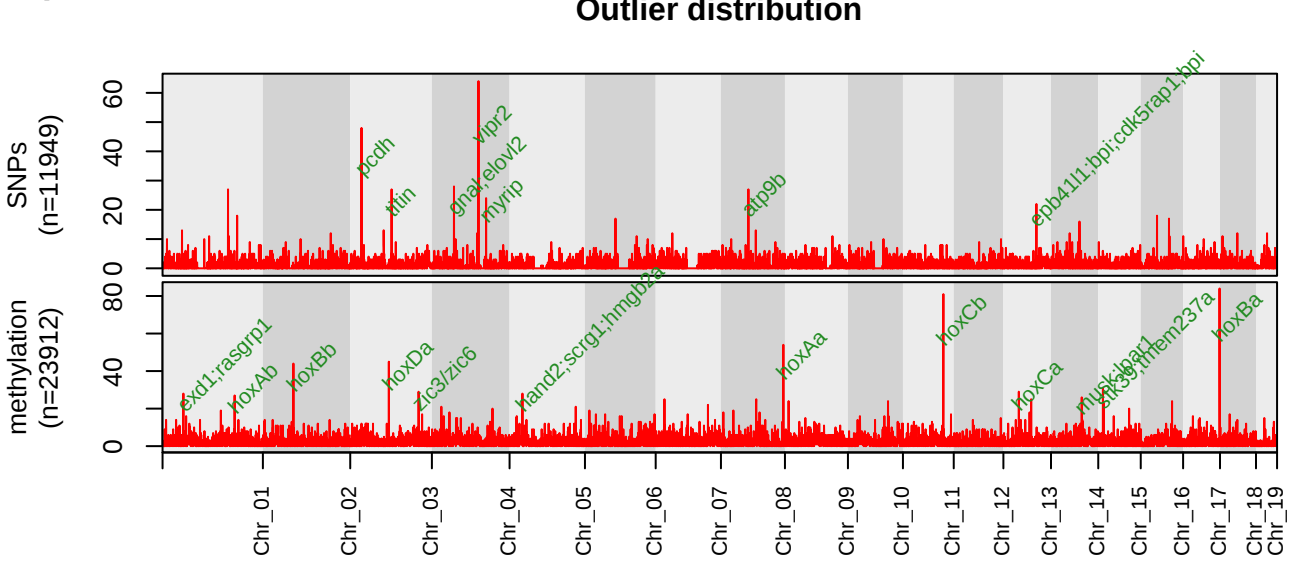


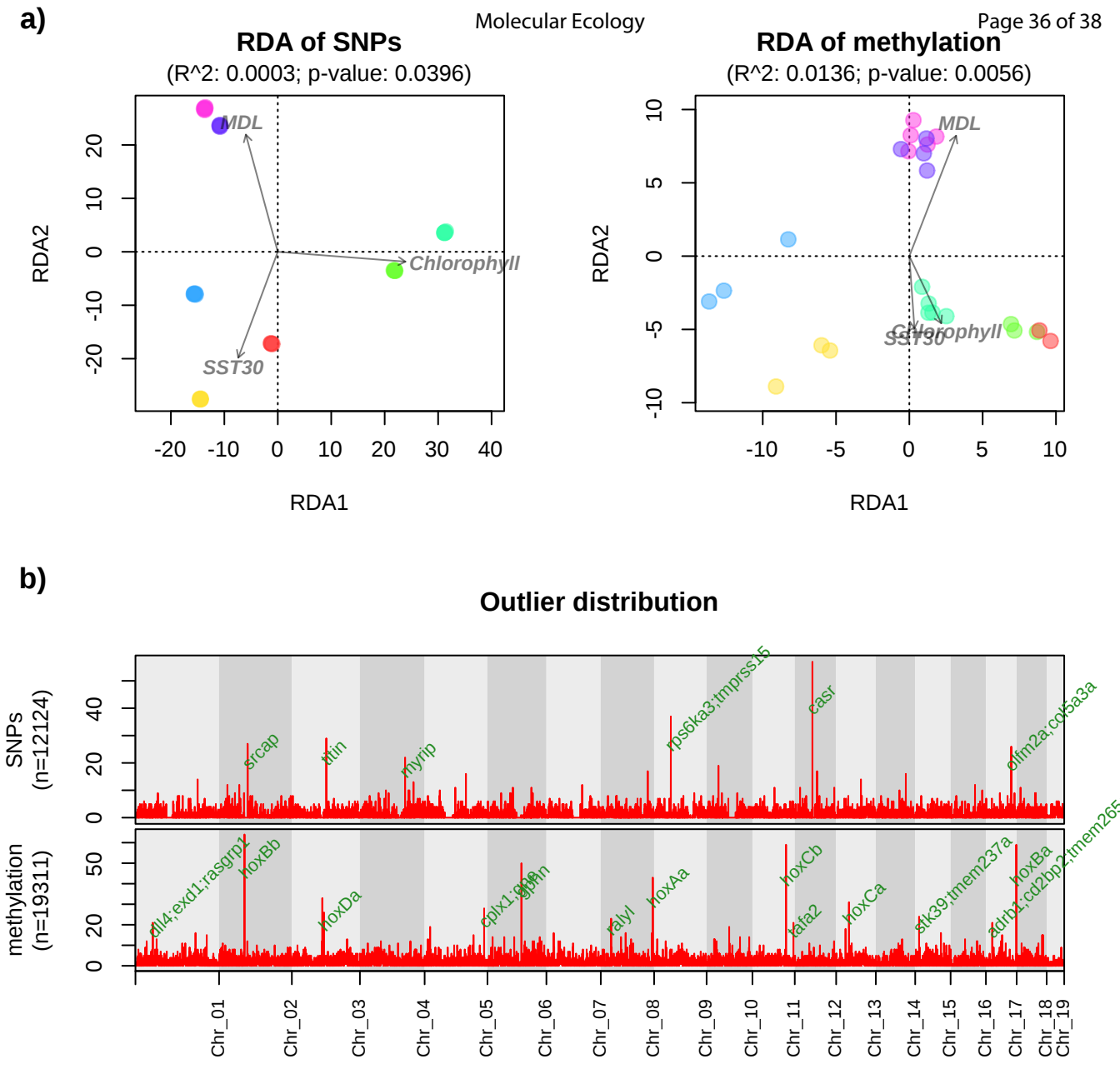

a)
exon_1
b)
intron_1
c)
promoter_1
d)
promoter_2
log10 (expression + 1e-7)
4
2
0
-2
-4
-6
Overall
[0, 0.2]
(0.2, 1]
Methylation

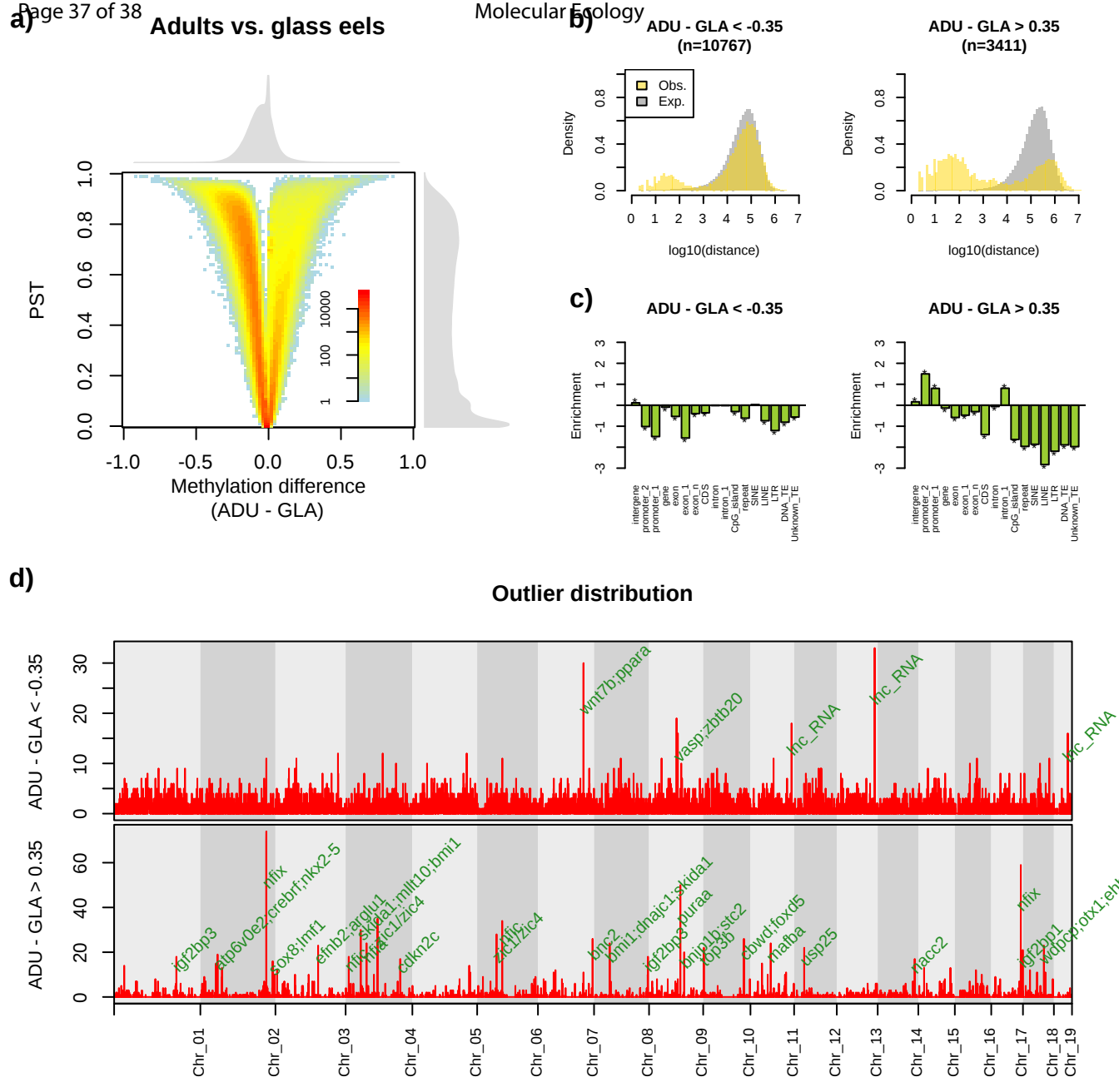
a)

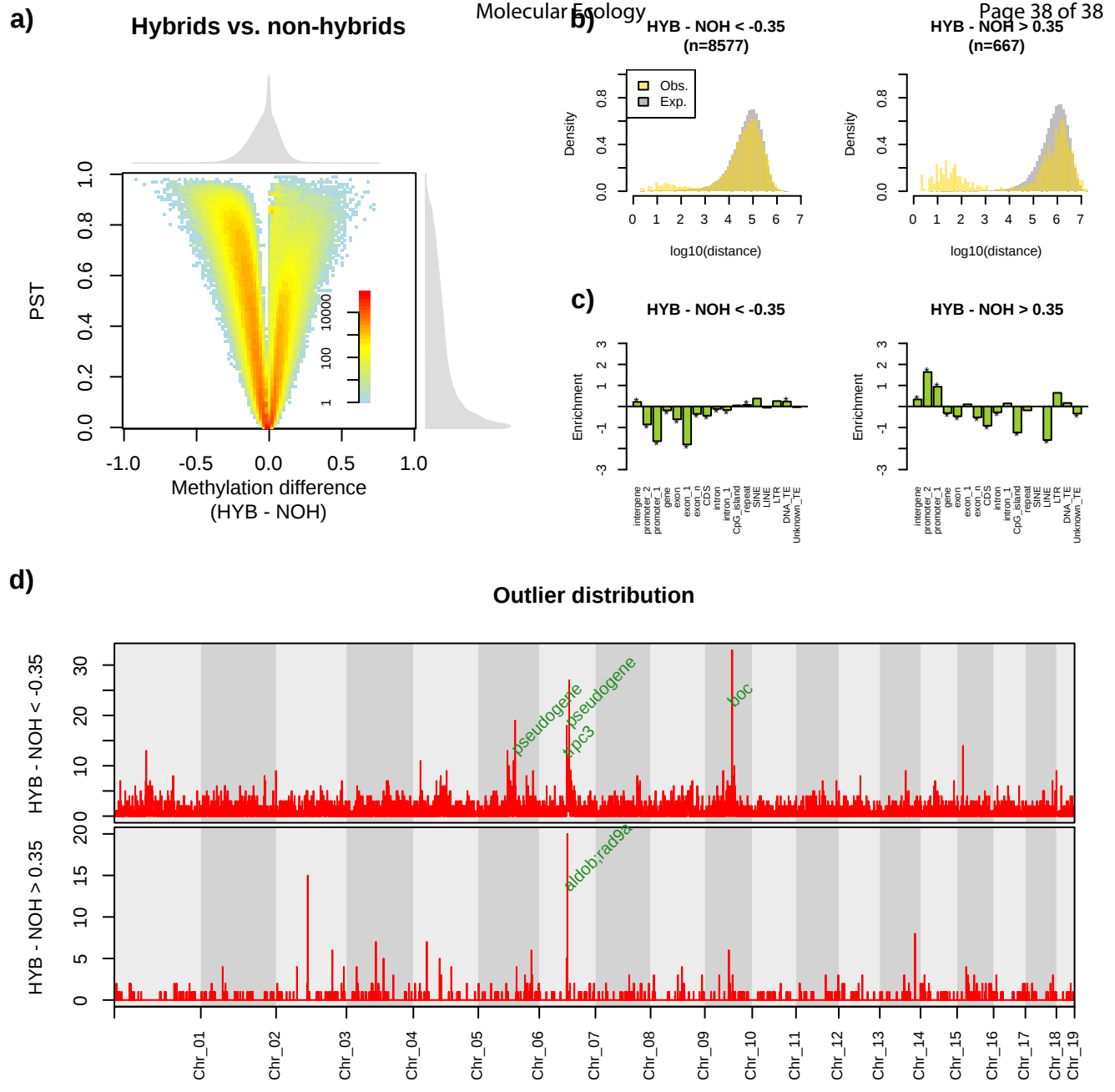

RDA of SNPs
(R^2: 0.0006; p-value: 0.0028)
RDA2
RDA1
Gir
Bur
Sto
Seb
Rin
Ell
RDA of methylation
(R^2: 0.0298; p-value: 0.0004)
RDA2
RDA1
Sto
Ell
Gir
Rin
Seb
Bur

b)


Outlier distribution
SNPs
(n=11949)
methylation
(n=23912)
pcdh
vtin
vipr2
gnai;elovl2
myrip
atp9b
epb41l1;bpi;cdk5rap1;bpi
exd1;rasgrp1
hoxAb
hoxBb
hoxDa
zic3/zic6
hand2;scrg1;hmgb2a
hoxAa
hoxCb
hoxCa
hoxBa
Chr_01
Chr_02
Chr_03
Chr_04
Chr_05
Chr_06
Chr_07
Chr_08
Chr_09
Chr_10
Chr_11
Chr_12
Chr_13
Chr_14
Chr_15
Chr_16
Chr_17
Chr_18
Chr_19


a)
RDA of SNPs
(R^2: 0.0003; p-value: 0.0396)
MDL
Chlorophyll
SST30
RDA2
RDA1
RDA of methylation
(R^2: 0.0136; p-value: 0.0056)
MDL
Chlorophyll
SST30
RDA2
RDA1
b)
Outlier distribution
SNPs
(n=12124)
methylation
(n=19311)
srcap
titin
myrip
rps6ka3;tmprss15
casr
olfm2a;col5a3a
dll4;exd1;rasgrp1
hoxBb
hoxDa
cplx1;gpm6a
hoxAa
hoxCb
tafa2
hoxCa
stk39;tmem237a
hoxBa
adrb1;cd2bp2;tmem265
Chr_01
Chr_02
Chr_03
Chr_04
Chr_05
Chr_06
Chr_07
Chr_08
Chr_09
Chr_10
Chr_11
Chr_12
Chr_13
Chr_14
Chr_15
Chr_16
Chr_17
Chr_18
Chr_19


a)
Adults vs. glass eels
PST
Methylation difference
(ADU - GLA)
b)
ADU - GLA < -0.35
(n=10767)
ADU - GLA > 0.35
(n=3411)
Obs.
Exp.
Density
log10(distance)
c)
ADU - GLA < -0.35
ADU - GLA > 0.35
Enrichment
intergene
promoter_2
promoter_1
gene
exon_n
exon_1
exon_n
CDS
intron
intron_1
CpG_island
repeat
SINE
LINE
LTR
DNA_TE
Unknown_TE
d)
Outlier distribution
ADU - GLA < -0.35
ADU - GLA > 0.35
wnt7b;ppara
lnc_RNA
lasp;zbtb20
lnc_RNA
lnc_RNA
lnc_RNA
nfix
igf2bp3
atp6v0e2;crebrf;nkx2-5
sox8;lmf1
efnb2;arglu1
nfix
skida1;mlht10;bmi1
nfa4c1/zic4
cdkn2c
zic1/zic4
lnc2
bmi1;dnajc1;skida1
igf2bp3;puraa
dnja1b;stc2
tp53b
cbwd;foxd5
nafba
dsp25
nacc2
nfix
igf2bp1
wdpcp;otx1;eh
Chr_01
Chr_02
Chr_03
Chr_04
Chr_05
Chr_06
Chr_07
Chr_08
Chr_09
Chr_10
Chr_11
Chr_12
Chr_13
Chr_14
Chr_15
Chr_16
Chr_17
Chr_18
Chr_19


a)
Hybrids vs. non-hybrids
PST
Methylation difference
(HYB - NOH)
b)
HYB - NOH < -0.35
(n=8577)
HYB - NOH > 0.35
(n=667)
Obs.
Exp.
Density
log10(distance)
c)
HYB - NOH < -0.35
HYB - NOH > 0.35
Enrichment
intergene
promoter_2
promoter_1
gene
exon_1
exon_n
CDS
intron
intron_1
CpG_island
repeat
SINE
LINE
LTR
DNA_TE
Unknown_TE
d)
Outlier distribution
HYB - NOH < -0.35
HYB - NOH > 0.35
pseudogene
pseudogene
xpc3
boc
aldob;radga
Chr_01
Chr_02
Chr_03
Chr_04
Chr_05
Chr_06
Chr_07
Chr_08
Chr_09
Chr_10
Chr_11
Chr_12
Chr_13
Chr_14
Chr_15
Chr_16
Chr_17
Chr_18
Chr_19