

# High nucleotide diversity accompanies differential DNA methylation in naturally diverging populations

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## Abstract

Epigenetic mechanisms such as DNA methylation (DNAm) are thought to comprise an invaluable adaptive toolkit in the early stages of local adaptation, especially when genetic diversity is constrained. However, the link between genetic diversity and DNAm has been scarcely examined in natural populations, despite its potential to shed light on the evolutionary forces acting on methylation state. Here, we analysed reduced-representation bisulfite sequencing and whole genome pool-seq data from marine and freshwater stickleback populations to examine the relationship between DNAm variation (between- and within-population), and nucleotide diversity in the context of freshwater adaptation. We find that sites that are differentially methylated between populations have higher underlying standing genetic variation, with diversity higher among sites that gained methylation in freshwater than those that lost it. Strikingly, while nucleotide diversity is generally lower in the freshwater population as expected from a population bottleneck, this is not the case for sites which lost methylation which instead have elevated nucleotide diversity in freshwater compared to marine. Subsequently, we show that nucleotide diversity is higher among sites with ancestrally variable methylation and also positively correlates with the sensitivity to environmentally induced methylation change. The results suggest that as selection on the control of methylation state becomes relaxed, so

1 too does selection against mutations at the sites themselves. Increased epigenetic variance in a  
2 population is therefore likely to precede genetic diversification.

3 **Keywords:** DNA methylation, stickleback, epigenetic, nucleotide diversity, local adaptation

#### 4 **Abbreviations:**

5 DNAm – DNA methylation

6 DM – Differential Methylation

7 DMC – Differentially Methylated Cytosine

8 DMR – Differentially Methylated Region

9 FW – Freshwater

10 FW-hyper – Hypermethylated in Freshwater population

11 FW-hypo – Hypomethylated in Freshwater population

12 Non-DMC – Non-Differentially Methylated Cytosine

13 RRBS – Reduced Representation Bisulfite Sequencing

14 SNP – Single Nucleotide Polymorphism

15

#### 16 **Introduction**

17 DNA methylation (DNAm) is an epigenetic mark whose roles in genome regulation, including  
18 gene expression regulation and transposable element suppression, have been well studied (He  
19 et al. 2011). Its role in local adaptation and long term evolution however, for example via  
20 plasticity, remains a topic of active debate. Methylome data support a potential role for DNAm  
21 in local adaptation in several species (Dubin et al. 2015; Sammarco et al. 2022), revealing that  
22 some genomic regions show differential methylation (DM) between different locally adapted  
23 populations. Populations (Johnson and Kelly 2020) and species (Vernaz et al. 2021) with low  
24 genetic divergence from one another have been found to differ considerably in DNAm patterns  
25 at environmentally relevant loci, raising the possibility that DNAm can be a source of  
26 phenotypic variation which increases adaptive potential of populations when genetic diversity is  
27 challenged (Flores et al. 2013). While such variation in DNAm can be environmentally induced  
28 or stochastic (Richards 2006), it is also influenced by genetic diversity. For example, DNAm is  
29 determined by the presence of sites with the capacity to be methylated (typically in a CpG  
30 context) and is subject to *trans*- and *cis*-regulation (Villicaña and Bell 2021). Therefore, the  
31 potential for methylation and corresponding plasticity is determined by the local genomic

1 context, and thus the 'epigenetic potential' of a population evolves at the sequence level (Kilvitis  
2 et al. 2017, see also Adrian-Kalchhauser et al. 2020).

3 It is also established that the epigenetic conformation of the genome affects the propensity for  
4 sequence change. Notably, DNAm can influence mutation rates due to the higher susceptibility  
5 of methylated Cs to spontaneous deamination to form thymine (Xia et al. 2012; Poulos et al.  
6 2017; Zhou et al. 2020). In mammals CpG mutation rates have been estimated at 10-50x higher  
7 than in other sequence contexts (Walser and Furano 2010). CpG mutation rate also has a  
8 nuanced relationship with methylation levels, as CpG sites with the highest mutation rates in  
9 human populations were observed to have low-to-intermediate methylation levels in cultured  
10 cells (Xia et al 2012). Therefore, by influencing sequence evolution, epigenetic variation may  
11 have unappreciated roles in the emergence of genomic novelties and adaptations (Storz et al.  
12 2019; Guerrero-Bosagna 2020), as well as mediating environmental influences on sequence  
13 evolution (Guerrero-Bosagna 2012; Lu et al. 2021).

14 Despite the interdependence of DNAm and sequence variation, the potential importance of this  
15 link in local adaptation has been largely overlooked. For example, typical workflows for  
16 detection of differential methylation tend to exclude CpG sites that are not detected at a certain  
17 coverage in a certain proportion of individuals (e.g. Akalin et al. 2012), and therefore it could be  
18 assumed that genetic diversity of those sites is irrelevant. However, these sites may  
19 nevertheless harbour genetic variants in the population, the relative frequencies of which may  
20 be informative about evolutionary forces acting on methylation state, potentially allowing further  
21 dissection of the manner in which DM evolves in the context of local adaptation. Indeed,  
22 methylation sites within certain promoters have already been shown to exhibit selective sweep  
23 signatures in *Arabidopsis* (Shirai et al. 2021). Epigenetic diversification is one of many possible  
24 routes to local adaptation (e.g. Smith et al. 2016) but may occur in conjunction with others, such  
25 as selection on discrete new mutations (hard sweeps) or on standing genetic variation (soft  
26 sweeps) (Bernatchez 2016; Hermisson and Pennings 2017). For example, if epigenetic  
27 modifications at multiple loci could confer similar adaptive benefit, epigenetic diversification  
28 could occur in conjunction with a soft sweep. Furthermore, given the heightened mutation rate  
29 of methylated Cs and its complex relationship with methylation levels (Xia et al, 2012),  
30 acquisition of methylation in a divergent population or a change in methylation level may  
31 influence mutation rates at affected sites. There is therefore a need to examine the relationships  
32 between differential methylation and nucleotide diversity in divergent populations.

1 The three-spined stickleback fish (*Gasterosteus aculeatus*) has long been a popular model to  
2 study the genetics and, more recently, the epigenetics, of local adaptation. Ancestrally a marine  
3 fish, *G. aculeatus* has repeatedly and rapidly colonised freshwater habitats over the last few  
4 millennia, with large waves of colonisations having occurred since the formation of glacial lakes  
5 following the last ice age (Jones et al. 2012; Roberts Kingman et al. 2021). Freshwater-adapted  
6 morphs show numerous phenotypic adaptations including the loss of armour plating (Barrett et  
7 al. 2008) and changes to kidney morphology (Hasan et al. 2017). Phenotypic and genetic  
8 divergence has been observed over short time scales (Lescak et al. 2015), with large shifts in  
9 frequencies of particular alleles having been observed over just a few years in newly  
10 established lake populations (Roberts Kingman et al. 2021). Such rapid fixation of alleles on the  
11 basis of standing genetic variation is characteristic of a soft sweep (Bernatchez 2016). However,  
12 the rapid adaptability and high plasticity of sticklebacks (Day et al. 1994) also makes the  
13 contribution of epigenetic variation to freshwater adaptation compelling. Multiple studies have  
14 used bisulfite sequencing to reveal differentially methylated sites in CpG context (DMCs) or  
15 differentially methylated regions (DMRs) between marine and freshwater populations. Some  
16 DMCs and DMRs are in the vicinity of genes relevant to freshwater adaptation (Smith et al.  
17 2015; Artemov et al. 2017; Heckwolf et al. 2020; Hu et al. 2021).

18 A potential role for epigenetic variation in freshwater adaptation is especially pertinent given that  
19 the formation of freshwater populations has been characterised by population bottlenecks,  
20 constraining genetic diversity. Steep declines in the effective population size  $N_e$  have been  
21 observed in newly established freshwater populations both from time series experiments  
22 (Aguirre et al. 2022) and ancient DNA (Kirch et al. 2021). Interestingly, in their comparison of gill  
23 DNA methylomes between marine and freshwater fish in the White Sea region, Artemov et al.  
24 (2017) showed that freshwater fish had higher variance of DNAm compared to marine fish, in  
25 line with the idea that higher epigenetic variation could compensate for reduced genetic  
26 diversity, enhancing the adaptive potential of a population following a bottleneck.

27 While genome and / or epigenome data have been generated from multiple stickleback  
28 populations across the Northern Hemisphere, the White Sea population complex is unique in  
29 that a variety of different data types have been generated from populations inhabiting the same  
30 region, including DNAm (Artemov et al. 2017), mRNA and small RNAseq (Rastorguev et al.  
31 2016; Rastorguev et al. 2017), and whole genome pool-seq (Terekhanova et al. 2014, 2019).  
32 Freshwater colonisation in this region has occurred relatively recently, with the oldest sampled  
33 lake estimated to have been formed approx. 700 years ago. While nucleotide diversity is

1 typically lower in freshwater populations, likely due to a past bottlenecks (Terekhanova et al.  
2 2014), patterns of nucleotide diversity at methylation sites, and DMCs specifically, have not  
3 been addressed. Shifts in nucleotide diversity at DMCs may be informative about the  
4 evolutionary forces acting on DNAm during local adaptation, namely the tightening or  
5 loosening of selective constraint on methylation state in a new environment.

6 Here, we combine epigenetic and genetic data from the White Sea stickleback population  
7 complex to study the interactions between methylation differences and nucleotide diversity  
8 during freshwater colonisation. We examined nucleotide diversity in relation to methylation  
9 divergence, variance, and the environmental inducibility of methylation state, considering both  
10 variance and inducibility as indicators of the relative stringency DNAm regulation.

## 11 **Results**

12 *Elevated nucleotide diversity accompanies differential methylation but level depends on the*  
13 *direction of methylation change*

14 For generation of DNAm and genome sequence data, respectively, both Artemov et al. and  
15 Terekhanova et al. sampled freshwater fish from the same lake (Lake Mashinoye) and marine  
16 fish from nearby coastal locations in the Kandalaksha gulf. A combined analysis of samples  
17 from these two datasets therefore allowed us to identify differentially methylated cytosines in  
18 CpG context (DMCs) between marine and freshwater populations and examine the nucleotide  
19 diversity of these sites in separate samples of those populations (**Fig. 1**). The RRBS data  
20 (Artemov et al. 2017) derived from gill tissue from a total of 11 individuals. These included six  
21 individuals as part of the main population comparison (N = 3 per population) and a further five  
22 experimental treatment animals that were used for a subsequent analysis of site inducibility.  
23 After filtering to remove sites with C-T/G-A SNPs detected in RRBS individuals, which could  
24 otherwise lead to spurious counts of unmethylated Cs, the analysis included just over 1 million  
25 CpG sites with at least 5x alignment coverage in all individuals, comprising approx. 6.9% of  
26 CpGs in the genome. The pool-seq data (Terekhanova et al. 2014, 2019) comprised sequenced  
27 material of two pools containing 12 marine and 10 freshwater individuals, and with genome  
28 coverage (high quality alignments) of 10.4x and 8x, respectively.

29 We first derived measures of nucleotide diversity of differentially methylated (DMCs) and non-  
30 differentially methylated DNAm sites (Non-DMCs) in the form of  $\pi$  (average number of pairwise  
31 differences within population) (Nei and Li 1979), Watterson's  $\theta$  (population-scaled mutation rate)  
32 (Watterson 1975), and Tajima's  $D$  (Tajima 1989).  $\pi$  and  $\theta$  are complementary measures of

1 within-population nucleotide diversity, while Tajima's  $D$  is a composite statistic derived from  
2 these two measures and can identify deviation of a sequence from neutrality. We also derived  
3 pairwise  $F_{st}$  (ratio of between-to-within population diversity; Weir and Cockerham 1984), which  
4 in a local sequence context can signify differential selection between populations. All nucleotide  
5 diversity measures were derived from pool-seq data at methylation sites detected from RRBS  
6 data (**Fig. 1**). Considering Non-DMCs as representing a baseline level of nucleotide diversity at  
7 cytosines in CpG context, we compared the  $\pi$  of Non-DMCs with that of DMCs that either lost  
8 methylation (at least 15% fewer methylated copies at  $p \leq 0.05$ , FW-hypo) or gained  
9 methylation in freshwater compared to marine (at least 15% more methylated copies at  $p \leq$   
10 0.05, FW-hyper). Substantially more FW-hypo sites (91,320) were detected than FW-hyper sites  
11 (14,508), while 895,121 sites were Non-DMCs. For nucleotide diversity analyses we  
12 subsampled Non-DMCs by taking one random Non-DMC within 2Mb of each DMC, resulting in  
13 a subsample of 99,585, approx. 11% of the total number. For each of these categories,  
14 nucleotide diversity measures were calculated per-chromosome.

15 We observed consistent associations between differential methylation and nucleotide diversity.  
16 DMCs showed elevated  $\pi$  compared to Non-DMCs regardless of population, with the highest  $\pi$   
17 observed amongst sites which gained methylation in FW (FW-hyper) (**Fig. 2A**). When  
18 comparing  $\pi$  between populations for each category of sites,  $\pi$  was slightly though significantly  
19 reduced in FW compared to marine at Non-DMCs (paired Wilcoxon test,  $p < 0.001$ ), reflecting  
20 the expected reduction in nucleotide diversity in the derived population.  $\pi$  calculated for sliding  
21 windows across chromosome 1 suggested that the pattern of reduced diversity in FW is a  
22 genome-wide feature (**Fig. S1A**) which likely resulted from a past bottleneck. FW  $\pi$  was  
23 similarly lower at FW-hyper sites ( $p < 0.001$ ), but not at FW-hypo sites which rather showed  
24 elevated  $\pi$  in FW compared to marine ( $p = 0.017$ ). Watterson's  $\theta$  showed a similar pattern to  
25 that of  $\pi$ , although there was no significant difference between marine and FW at FW-hypo sites  
26 (**Fig. S2**). Between-population diversity ( $F_{st}$ ), showed significant differences between DMCs  
27 and Non-DMCs, with both FW-hypo and FW-hyper sites showing significantly higher  $F_{st}$  values  
28 than Non-DMCs (paired  $t$ -tests,  $p < 0.01$  in both cases; **Fig. 2B**). Tajima's  $D$  was mostly  
29 negative in both populations as indicated by sliding windows across chromosome 1 (**Fig. S1B**),  
30 but was higher in FW, consistent with the scenario of a population bottleneck following  
31 freshwater colonisation. Tajima's  $D$  of methylation sites largely reflected this chromosome-wide  
32 pattern of higher values in FW, with the exception of FW-hyper sites which showed no  
33 significant difference in Tajima's  $D$  between the two populations (**Fig. 2C**). Tajima's  $D$  tended to  
34 be higher among DMCs than Non-DMCs in marine, but not in FW.

1 These patterns of elevated nucleotide diversity were not driven by enrichment for DMCs in  
2 regions of high diversity. Rather, elevated  $\pi$  of DMCs was found to be strongly localised around  
3 individual DMCs (**Fig. S3**). The pattern was largely consistent across different genomic features  
4 including CpG islands, gene bodies, promoters, and intergenic regions (**Fig. S3**). No clear  
5 pattern of localised elevated diversity was observed for DMCs which fell within differentially  
6 methylated regions (DMRs) (**Fig. S3**), however only a fraction of FW-hypo (approx. 17%) and  
7 FW-hyper DMCs (approx. 12%) fell within DMRs.

8 Next, we determined which mutation type(s) were most likely to be driving the elevated  $\pi$  of  
9 DMCs, and specifically whether this was driven by an over-abundance of C-T transitions. To this  
10 end, the percentages of sites in each category harbouring biallelic SNPs of different types (C-  
11 T/G-A, C-A/G-T, or C-G/G-C) were calculated from the pool-seq data. The majority of SNPs  
12 were C-T/G-A, comprising 90% of SNPs across all categories in marine and 94% in freshwater.  
13 The proportion of sites harbouring biallelic C-T/G-A SNPs across the three categories of  
14 methylation site and two populations showed a similar pattern to that of  $\pi$ , with FW-hyper sites  
15 harbouring the highest proportion of C-T/G-A SNPs in both populations (**Fig. 2D**). Marine had  
16 more C-T/G-A SNPs than freshwater in the Non-DMC (paired Wilcoxon test,  $p < 0.001$ ) and FW-  
17 hyper categories ( $p < 0.001$ ) but not the FW-hypo category, in which FW and marine had similar  
18 proportions of C-T/G-A SNPs. Meanwhile, the percentage of other SNP types showed no clear  
19 differences between the site categories. Therefore, the generally increased nucleotide diversity  
20 amongst DMCs relative to Non-DMCs seemed to be driven by a greater occurrence of C-T  
21 mutations.

### 22 *Higher nucleotide diversity of infrequently-methylated DMCs*

23 The finding that sites which gained methylation in freshwater (FW-hyper) had the highest  $\pi$  and  
24 highest proportion of C-T/G-A mutations in marine (**Fig. 2**) was contrary to expectations, as  
25 these sites would be expected to be infrequently methylated in marine and therefore not at high  
26 risk of mutation via deamination. We therefore tested the relationship between  $\pi$  and the  
27 distributions of mean percentage of methylation (hereafter Mean{PM}) across the three site  
28 categories. Here, Mean{PM} refers to the average percentage of copies on which the C is  
29 methylated, or in other words the average frequency of the methylation mark among copies of  
30 given CpG site. We found that Non-DMCs displayed a bimodal density distribution, with most  
31 sites either very frequently (>75%) or very infrequently methylated (**Fig. 3A**, left). Meanwhile,  
32 the distributions of Mean{PM} of DMCs were markedly different to those of Non-DMCs. FW-  
33 hypo sites were characterised by a shift from mostly high Mean{PM} in marine to mostly

1 intermediate Mean{PM} in FW (**Fig. 3A**, middle). Mirroring this pattern, FW-hyper sites were  
2 characterised by a shift from low-intermediate Mean{PM} in marine to high Mean{PM} in FW  
3 (**Fig. 3A**, right).

4 Because pool-seq data are not appropriate for estimating  $\pi$  at the level of a single site, we used  
5 a ranking procedure to examine the relationship between Mean{PM} and  $\pi$ . Sites were divided  
6 into ranks according to their Mean{PM}, with higher ranks containing sites with higher  
7 Mean{PM}. This ranking was performed separately for each population and each site category,  
8 and a measure of  $\pi$  obtained for each rank. The relationship between the rank-level Mean{PM}  
9 and  $\pi$  was clearly non-monotonic for Non-DMCs, with the highest values appearing at low to  
10 intermediate Mean{PM} of around 25% (**Fig. 3B**, left). The higher  $\pi$  of FW-hypo sites in  
11 Freshwater appeared to be driven largely by sites in the low-intermediate range (**Fig. 3B**,  
12 middle). Amongst FW-hyper sites, those with the highest Mean{PM} clearly contributed to the  
13 lower  $\pi$  of these sites in FW (**Fig. 3B**, right).

14 We also examined the relationship of  $\pi$  with the population difference in Mean{PM} (i.e. the  
15 extent of hypo- or hypermethylation). We observed that among FW-hypo sites, the freshwater  
16 population had the largest increases in the  $\pi$  where the hypomethylation was strongest (**Fig. 3C**  
17 and **Fig. 3D**). FW-hyper sites also increased in  $\pi$  with the extent of hypermethylation (**Fig. 3C**),  
18 but this also corresponded with greater loss of  $\pi$  in FW (**Fig. 3D**). Meanwhile, sites with larger  
19 difference in Mean{PM} in either direction (methylation loss or gain) had higher  $F_{st}$  (**Fig. 3E**).

#### 20 *High nucleotide diversity accompanies high variability in ancestral methylation*

21 Considering that sites with intermediate Mean{PM} are liable to have more variable methylation  
22 frequency than those with very low or very high Mean{PM}, we also considered the relationship  
23 between  $\pi$  and the standard deviation of percentage methylation (hereafter  $SD_{meth}$ ). We  
24 predicted that sites with more variable methylation would have higher nucleotide diversity,  
25 reasoning that the methylation state of these sites is not stringently controlled and therefore  
26 mutations at these sites may have little impact on function. We first examined the distributions of  
27  $SD_{meth}$  values of sites in the Non-DMC, FW-hypo and FW-hyper categories. Non-DMCs were  
28 largely invariable, with slightly more variable methylation in freshwater compared to marine (**Fig.**  
29 **4A**, left), consistent with the observations of Artemov et al. (2017). FW-hypo sites were  
30 characterised by a pronounced increase in  $SD_{meth}$  from ancestral to derived population, shifting  
31 from a left-skewed distribution in marine to a Gaussian-like distribution in freshwater (**Fig. 4A**,



1 middle). Meanwhile, FW-hyper sites, showed a skewed distribution in marine with an elongated  
2 plateau to the right, while methylation was less variable in freshwater (**Fig. 4A**, right).

3 To examine the relationship between  $\pi$  and the variability in methylation, sites were ranked  
4 according to  $SD_{\text{meth}}$  in each population and site category. We observed that  $\pi$  increased steeply  
5 at an  $SD_{\text{meth}}$  above  $\sim 15$  in all three site categories in the marine population and two of the three  
6 site categories in freshwater (**Fig. 4B**). For the  $\pi$  of FW-hypo sites in FW however, there was no  
7 obvious relationship with the exception of two ranks showing highly elevated  $\pi$  at opposite ends  
8 of the range of  $SD_{\text{meth}}$  values (**Fig. 4B**, middle). The high  $\pi$  of the lowest rank, which  
9 contradicted the trend observed in the other categories, is likely attributable to high ancestral  
10 diversity of sites that have almost completely lost methylation in freshwater (and therefore attain  
11 very low variance) but retain high ancestral nucleotide diversity. The relationship between  $\pi$  and  
12 the shift in  $SD_{\text{meth}}$  would appear to support this notion because sites with the largest decrease in  
13  $SD_{\text{meth}}$  in freshwater also have the highest  $\pi$  in marine (**Fig. 4C**). Shifts in the  $SD_{\text{meth}}$  were also  
14 associated with shifts in  $\pi$  (**Fig. 4D**). Both amongst FW-hypo and FW-hyper sites, there was a  
15 trend towards reduced  $\pi$  with decreased  $SD_{\text{meth}}$  and increased  $\pi$  with increased  $SD_{\text{meth}}$ . For FW-  
16 hypo sites this was only apparent at extreme shifts in  $SD_{\text{meth}}$ , while for FW-hyper sites there was  
17 a significant linear relationship (linear model,  $R^2 = 0.58$ ,  $p < 0.001$ ). No clear relationships were  
18 observed between the shift in  $SD_{\text{meth}}$  and  $F_{\text{st}}$  for either FW-hypo or FW-hyper categories (**Fig.**  
19 **4E**).

### 20 *Environmentally inducible DNA methylation is linked with higher nucleotide diversity*

21 So far, we have considered differential methylation in regard to losses or gains of methylation  
22 that have been detected in a population  $\sim 700$  years after its colonisation of a new environment.  
23 While such differences may result from evolution, differences in methylation state can also be  
24 directly induced by the environment. Such inducibility may be important for adaptation but also  
25 subject to genetic variation. We therefore analysed additional samples from the dataset by  
26 Artemov et al. (2017), as in addition to the main population comparison, the authors also  
27 quantified gill methylation differences in fish from each population in response to a change in  
28 environmental salinity. Fish from each population were exposed to the opposite conditions, with  
29 marine fish exposed to reduced salinity and freshwater fish exposed to increased salinity (**Fig.**  
30 **5A**). Here, we reasoned that environmental inducibility of methylation state could also reflect the  
31 degree of genetic versus environmental control of methylation state, similar to increased  $SD_{\text{meth}}$   
32 possibly reflecting relaxed control of methylation state. Sites whose methylation state is more  
33 responsive to the environment could be assumed to be under looser genetic control and

1 possibly relaxed selection. In total, sites that were induced in either population constituted 3.8%  
2 of Non-DMCs, 11.3% of FW-hypo and 39.2% of FW-hyper sites, with DMCs, and particularly  
3 FW-hyper sites therefore being enriched for induced sites. When considering the proportions of  
4 induced sites in each population separately (**Fig. 5B**), freshwater had a higher proportion of  
5 induced sites than marine among FW-hypo sites and a lower proportion among FW-hyper sites,  
6 a pattern that almost perfectly mirrored that which was observed for  $\pi$  (**Fig. 2A**). Induced sites  
7 were slightly enriched amongst CpG islands in that a higher % of induced sites than non-  
8 induced sites resided in CpG islands (Wilcoxon tests,  $p < 0.01$  for both FW-hypo and FW-  
9 hyper), while among FW-hypo sites induced sites were slightly more likely than non-induced  
10 sites to reside in promoter regions ( $p = 0.004$ ) (**Fig. S4**).

11 Sites that were induced in either population, or that were induced only in one of the two  
12 populations, had elevated  $\pi$  compared to sites that were not induced in either population (paired  
13  $t$ -tests, all  $p < 0.01$ ), while the  $\pi$  of non-induced DMCs was closer to that of Non-DMCs (**Fig.**  
14 **5C**). Furthermore, the gain in  $\pi$  in freshwater among FW-hypo sites appeared to be driven by  
15 sites that had gained inducibility (i.e. which were not ancestrally inducible), as this elevated  $\pi$   
16 was observed among sites that were induced only in freshwater (paired  $T$ -test,  $p < 0.001$ ), but  
17 not sites that were induced only in marine ( $p = 0.83$ ). Meanwhile, amongst FW-hyper sites,  $\pi$   
18 was consistently significantly lower in freshwater compared to marine (paired  $t$ -tests,  $p < 0.05$ ),  
19 with the exception of sites induced only in FW in which the difference was not significant ( $p =$   
20  $0.09$ ). When induced sites in each population were assigned to ranks according to the mean  
21 absolute induced methylation change (i.e. regardless of whether salinity change induced lower  
22 or higher methylation),  $\pi$  increased linearly with the mean induced methylation change in both  
23 marine (linear model,  $R^2 = 0.83$ ,  $p < 0.001$ ) and FW populations ( $R^2 = 0.59$ ,  $p < 0.001$ ) (**Fig. 5D**),  
24 suggesting that environmental inducibility can reliably predict nucleotide diversity.

25 Compared to non-induced sites, sites that were induced in either population had significantly  
26 elevated pairwise  $F_{st}$  in both FW-hypo (paired  $t$ -test,  $p < 0.001$ ) and FW-hyper categories ( $p =$   
27  $0.002$ ) (**Fig. 5E**). However, this elevated  $F_{st}$  was driven by sites that were induced in FW, as  
28 sites induced only in FW had significantly elevated  $F_{st}$  in both FW-hypo ( $p < 0.001$ ) and FW-  
29 hyper categories ( $p = 0.008$ ), while sites induced only in marine fish did not show elevated  $F_{st}$   
30 compared to non-induced sites. Indeed, FW-hypo sites that were induced only in marine even  
31 had lower  $F_{st}$  than non-induced sites ( $p = 0.046$ ). The elevated  $F_{st}$  of DMCs (as seen in **Fig.**  
32 **2B**) was therefore likely driven by sites that are inducible in the freshwater population. Finally,  
33 when induced sites in each population were ranked according to the degree of induced

1 methylation change in that population, sites that were induced in the freshwater population  
2 showed a weak positive correlation between inducibility and  $F_{st}$  (linear model,  $R^2 = 0.25$ ,  $p <$   
3  $0.001$ ), while sites that were induced in the marine population did not show such a correlation  
4 ( $R^2 = -0.01$ ,  $p = 0.4$ ) (**Fig. 5F**).

## 5 **Discussion**

6 Here, we examined the relationship between DNA methylation differences and nucleotide  
7 diversity in an ancestral and a derived population of wild three-spined stickleback. For this  
8 purpose, CpG sites with different methylation status in freshwater compared to the marine  
9 population were interpreted as changes in DNAm that occurred in the course of freshwater  
10 colonisation. Our analyses show that genetic diversity is intimately linked to variation in DNAm  
11 across both long (population differentiation) and short timescales (environmental responses).  
12 This link between DNAm and nucleotide diversity can shed light on the evolutionary forces  
13 acting on methylation state and could hint at the extent to which epigenetic changes precede  
14 sequence evolution.

15 *Sites prone to methylation divergence have high standing genetic variation driven by*  
16 *hypermutability of 5mC*

17 Despite applying stringent filtering to retain only CpG sites that were detected in all RRBS  
18 individuals (requiring all individuals to be either C/C or heterozygous at reference CpG loci), we  
19 found that not only did DMCs harbour SNPs among the individuals represented in the pooled  
20 sequencing dataset, but were even enriched for them (**Fig. 2A, D**). The high nucleotide diversity  
21 of DMCs was regardless of population, indicating that differential methylation occurred at sites  
22 of high standing genetic variation. Consistent with a probable past bottleneck (Terekhanova et  
23 al. 2014), nucleotide diversity was reduced in FW, a pattern that held for FW-hyper sites despite  
24 the expectation that sites gaining methylation should incur higher mutation rates (Xia et al.  
25 2012). However, FW-hypo sites – those that had lost methylation in freshwater – exhibited a  
26 slight increase in  $\pi$  in FW compared to marine, implying relaxed selection among these sites in  
27 the derived population.

28 The use of various measures of genetic diversity in parallel can provide insights into possible  
29 evolutionary processes at a fine scale. DMCs had higher diversity than Non-DMCs as measured  
30 by both  $\pi$  (**Fig 2A**) and  $\theta$  (**Fig. S2**), indicating both more pairwise differences at polymorphic  
31 sites and more polymorphic sites overall amongst these sites. However, DMCs also had higher  
32 Tajima's  $D$  (higher proportion of intermediate-frequency alleles) than Non-DMCs within the

1 marine population (**Fig. 2C**), suggesting that sites with a tendency to diverge in methylation  
2 state are those that were already under weaker selective constraint or possibly under balancing  
3 selection (Jackson et al. 2015). The generally higher Tajima's  $D$  in FW was expected following a  
4 recent population bottleneck (Stajich and Hahn 2005). However, this increase was not observed  
5 at FW-hyper sites, suggesting that while FW had an overall tendency to accumulate  
6 intermediate frequency alleles, this was impeded at FW-hyper sites, possibly due to increased  
7 selective constraint. The elevated  $F_{st}$  (**Fig. 2B**) of FW-hypo and FW-hyper sites compared to  
8 Non-DMCs further suggests that both types of DMC are subject to some degree of differential  
9 selection.

10 DNAm and mutations rates are intrinsically linked by the hypermutability of 5mC. Indeed, we  
11 find that the patterns of nucleotide diversity are driven by higher abundance of C-T/G-A SNPs  
12 amongst DMCs, but not other SNP types (**Fig. 2D**), suggesting that they are driven by higher  
13 rates of spontaneous deamination of methylated Cs (Xia et al. 2012). The hypermutability of  
14 5mC may further explain why FW-hypo sites were >6x more common than FW-hyper sites. If  
15 sites that acquire methylation during the transition to a new environment are more prone to  
16 mutation, then many such methylation gains would be transient. Thus, stable gains in  
17 methylation would be more difficult to attain than stable losses, and active selection might be  
18 required for their maintenance. Indeed, as sites with newly gained methylation must escape  
19 deamination in several individuals in order to be detected in the differential methylation analysis  
20 (due to stringent site filtering), FW-hyper sites may be enriched for the subset of new  
21 methylations that are under selective constraint.

22 We also note that while  $\pi$  was increased in FW relative to marine at FW-hypo sites (**Fig. 2A**),  
23 this increase was not observed in  $\theta$  (**Fig. S2**) or the percentage of C-T/G-A SNPs (**Fig. 2D**),  
24 which instead showed similar values in marine and FW. Nevertheless, given the lower overall  
25 diversity of FW, this population would need to have incurred elevated mutation rates at FW-  
26 hypo sites in order to reach similar values of  $\theta$  and C-T/G-A SNPs to the marine population.  
27 Therefore the slightly elevated  $\pi$  in FW is likely to have been driven largely by accumulation of  
28 C-T/G-A SNPs.

29 Overall, contrasting the different measures of nucleotide diversity reveals complex patterns of  
30 sequence evolution at methylation sites, reflecting ancestral standing genetic variation and  
31 possibly recent changes in the fitness landscape of methylation sites.

1 *Relationships between nucleotide diversity and methylation frequencies further imply differential*  
2 *selection of DMCs*

3 The relative frequencies at which sites are methylated (expressed as Mean{PM}, **Fig. 3**) capture  
4 both intra- and inter-individual variation in methylation state. Although the RRBS data derived  
5 from a specific tissue – gill, such a tissue is nevertheless heterogeneous, comprising of different  
6 specialised cell types (Pan et al. 2022). Very high or very low Mean{PM} values are therefore  
7 likely to comprise sites where the same state is consistently maintained across the majority of  
8 cells and/or cell types. Although cell type-specific methylation is likely to be important in some  
9 contexts (Loyfer et al. 2023), it could nevertheless be inferred that sites with consistent  
10 methylation state are more likely to be selectively constrained. We observed that among Non-  
11 DMCs, most sites had either very high or very low Mean{PM}, suggesting most sites have a  
12 methylation state that is consistently maintained across cell types (i.e. consistently methylated  
13 or non-methylated). Non-DMCs with intermediate Mean{PM} had higher  $\pi$  than those with very  
14 high or very low Mean{PM}, again indicating stronger selective constraints on sites which are  
15 consistently either methylated or non-methylated. This is consistent with previous observations  
16 that sites in the human genome with low to intermediate methylation frequency *in vitro* have  
17 higher mutation rates in human populations (Xia et al. 2012). We found that in sticklebacks,  
18 differential methylation in the freshwater population was characterised by shifts either towards  
19 (FW-hypo) or away from (FW-hyper) intermediate Mean{PM} (**Fig. 3A**), corresponding with  
20 increase or decrease in  $\pi$ , respectively (**Fig. 3B**). Accordingly,  $\pi$  increased in freshwater with  
21 the degree of hypomethylation and decreased with the degree of hypermethylation, while  $F_{st}$   
22 tended to increase with the degree of difference in either direction (**Fig. 3D, E**). Combined,  
23 these patterns imply that differential methylation occurs alongside differential selection, in that  
24 (stable) gains in methylation tend to be selectively constrained while sites that lose methylation  
25 are released from selective constraint. This would make sense given that a loss of methylation  
26 relinquishes the requirement of the locus to remain as a CpG dinucleotide.

27 *Genetic variation reflects (ancestral) epigenetic variation*

28 Inter-individual variability of DNA methylation remains understudied in natural populations, and  
29 yet it may hint at the processes by which the methylome evolves. Using the same RRBS  
30 dataset, Artemov et al. (2017) previously showed that methylation was more variable in the  
31 derived freshwater population. Here, we show that this effect depends on differential  
32 methylation (**Fig. 4A**). Indeed, the higher variability in freshwater appeared to be driven largely  
33 by FW-hypo sites which showed a Gaussian-like distribution of  $SD_{meth}$  values in the FW

1 population compared to a strongly left-skewed distribution of the same sites in marine. FW-  
2 hyper sites on the other hand became less variable in FW. Loss of methylation in FW therefore  
3 appears to be characterised by relaxed control of methylation state, while gain of methylation is  
4 associated with tighter control. Indeed, heightened variability of FW-hypo sites suggests that  
5 loosening of regulation is itself the cause of methylation loss.

6  $\pi$  tended to increase with variability of methylation (**Fig. 4B**), which would support the  
7 hypothesis that sites with less tightly maintained methylation state are under weaker selective  
8 constraint. This pattern was consistent across all three site categories in the marine population,  
9 but was absent among FW-hypo sites in FW. Genetic variation therefore reflects ancestral, but  
10 not recently acquired variability in methylation state. Relaxed selection on sites which lose  
11 methylation would lead to an accumulation of C-T mutations, while stronger selective constraint  
12 would reduce the nucleotide diversity of sites with stable methylation gain. Concordantly, sites  
13 that became less variable in FW tended to lose  $\pi$ , while sites that became more variable tended  
14 to gain  $\pi$  (**Fig. 4D**). The pattern was more prominent among FW-hyper sites which  
15 predominantly had both decreased  $SD_{\text{meth}}$  of methylation and decreased  $\pi$ . Among FW-hypo  
16 sites, the lack of correlation between  $SD_{\text{meth}}$  and  $\pi$  in freshwater (or difference in  $SD_{\text{meth}}$  and  
17 difference in  $\pi$ ) could be explained by the relatively young age of the freshwater population  
18 (~700 years) and subsequent lack of time for mutations to accumulate.

19 While it is plausible that the increased  $\pi$  and  $SD_{\text{meth}}$  of FW-hypo sites reflect relaxed selection  
20 on the regulation of methylation state, an alternative hypothesis is that variable loss of  
21 methylation reflects epigenetic responses that have occurred only in a fraction of individuals in  
22 the population. This scenario would also be consistent with elevated  $\pi$  of the FW-hypo sites; if  
23 these differential epigenetic responses were genotype-specific, the elevated  $\pi$  would reflect  
24 standing genetic variation as opposed to new mutations. Such a scenario would be consistent  
25 with a soft sweep (Hermisson and Pennings 2017), in which selection could act on many  
26 different genetic and epigenetic loci, thus maintaining diversity at both levels.

### 27 *Environmental inducibility of DNA methylation may predict sequence evolution*

28 The potential importance of epigenetic mechanisms in mediating plastic responses has long  
29 been discussed (Johnson and Tricker 2010) and, more recently, demonstrated experimentally  
30 (Stajic et al. 2019). Although the environmental induction of a particular epigenetic state (e.g.  
31 addition or removal of DNAm) can occur in the context of adaptive mechanisms (Lämke and  
32 Baurle 2017), such an induction may not necessarily constitute an adaptive response (see also

1 Hu and Barrett 2022). As such, we considered environmental inducibility in a different context, in  
2 that the degree of environmental inducibility of methylation state is (inversely) indicative of the  
3 degree of intrinsic regulation. We therefore use the term 'inducibility' loosely to refer to the  
4 sensitivity of a site to methylation change in response to the environment, regardless of its  
5 potential adaptive importance. We found that elevated  $\pi$  of and Fst of DMCs was driven by sites  
6 that were environmentally inducible (**Fig. 5A, B, E**), further supporting a hypothesis of relaxed  
7 regulation and relaxed selective constraint at sites that are responsive to environmental  
8 conditions. Furthermore, the increased  $\pi$  among FW-hypo sites in FW relative to marine was  
9 driven by sites that were induced only in FW, i.e. those not induced in the ancestral population,  
10 suggesting that nucleotide diversity is more likely to accumulate at sites where intrinsic control  
11 of methylation is relaxed (and therefore more sensitive to the environment). Indeed, the positive  
12 correlation between  $\pi$  and the degree of inducibility (**Fig. 5C**) suggests that the more sensitive  
13 the methylation state is to the environment, the more likely mutations are to be selectively  
14 neutral. Therefore, shifts in inducibility (in addition to shifts in methylation variance, as discussed  
15 above) may precede shifts in nucleotide diversity. Our results suggest that the majority of  
16 environmentally inducible sites are simply 'blowing in the wind' and do not have important  
17 functions for plasticity which would constrain nucleotide diversity. Nevertheless, in their analysis  
18 of Baltic Sea sticklebacks, Heckwolf et al. (2020) observed that the Fst of induced sites (marine  
19 fish responsive to lower salinity) depended on the direction of the induced change. Sites that  
20 were induced to the 'evolved' methylation state observed in the derived freshwater population  
21 had lower Fst than those that were induced in the opposite direction. This suggests that some  
22 environmentally inducible sites are indeed constrained by selection due to the importance of site  
23 plasticity. Here, we did not consider the direction of inducible change, merely considering  
24 inducibility as a proxy for the relative weakness of intrinsic regulation.

25 Again, these observations could also be reconciled with the scenario of a soft sweep, as it is  
26 also possible that plasticity of only some methylation sites is necessary to confer adaptation. In  
27 other words, plasticity of multiple sites provides multiple alternate routes to adaptation. As many  
28 methylation sites would therefore be redundant, they could be lost to mutation without  
29 detrimentally affecting the organism's capacity for adaptive plasticity.

### 30 *Limitations and future directions*

31 Our analyses have revealed striking associations between genetic and epigenetic variation in  
32 divergent stickleback populations. However, we must acknowledge limitations including the

1 extent of the data used to address the question, technical and analytical limitations, and  
2 knowledge gaps that pave the way for future investigations.

3 A key limitation is that the RRBS data we used came from only a single tissue type (gill), and  
4 therefore we were unable to determine which methylation differences between populations are  
5 tissue-specific and which are organism-wide. However, many divergent methylation states are  
6 not tissue specific, as a recent analysis of divergent cichlid ecotypes showed that a high  
7 proportion of DMRs were shared across tissue types (Vernaz et al. 2021). Also, with respect to  
8 the studied divergence between marine and freshwater environments, gills are key to salt  
9 homeostasis and their ability to respond to changes in osmolarity affects the entire organism. A  
10 similar sampling limitation is that, while the two datasets used in this study included marine fish  
11 collected from similar locations in the White Sea, we cannot be sure that pool-seq marine and  
12 RRBS marine individuals were representative of the same population. In a structured  
13 population, DNAm and nucleotide variation may co-vary at different sites across different  
14 branches of the population. This would cause some relationships to be missed if RRBS and  
15 pool-seq individuals came from different branches of the population.

16 The detection of differential methylation is highly sensitive to the analytical methods applied.  
17 Firstly, the use of RRBS instead of whole-genome bisulfite sequencing limited the number of  
18 sites that could be analysed to approx. 6.9% of genome-wide CpGs. It is also selective for CG-  
19 rich regions, and as such approx. half of the analysed sites belonged to CpG islands (see **Fig.**  
20 **S3**). Therefore, the patterns we observe may not necessarily be representative of genome-wide  
21 patterns. RRBS remains however a powerful and cost-effective means of methylome  
22 interrogation (see also Klughammer et al. 2023). Secondly, the retention of individual-specific  
23 CpG sites can lead to the detection of differential methylation simply due to differences in the  
24 abundance of CpGs available to be methylated – i.e. directly due to SNPs (Wulfridge et al.  
25 2019). We suggest that whether or not individual-specific CpGs are retained in an analysis, and  
26 by extension the definition of 'differential methylation', should depend on the goals of the study.  
27 Here, by excluding individual-specific sites we aimed to detect differential methylation that arose  
28 through the differential action of the methylation machinery and not due directly to nucleotide  
29 variation at the sites themselves. We acknowledge that excluding individual-specific CpGs risks  
30 ignoring a potentially high proportion of methylation variation and, while it was not the goal of  
31 this study to extensively characterise this variation, the results should be interpreted with this in  
32 mind. A third important analytical limitation stems from the necessity to filter C-T/G-A SNPs from  
33 bisulfite sequencing data to avoid A bases resulting from these SNPs being mis-classed as



1 unmethylated Cs that were bisulfite-converted to Ts. Although we used a combination of three  
2 SNP-callers designed for BS-seq data (see methods), we cannot be certain that some  
3 differential methylation was not the result of SNPs that these algorithms failed to detect (see  
4 Lindner et al. 2022).

5 In a broader context, our study is limited in that we only examined one population pair. It is  
6 therefore currently not known whether the patterns we observed occur more broadly across  
7 different local adaptations (in stickleback and other species) or whether they are idiosyncratic to  
8 the relatively recent colonisation event considered in this study (~700 years). The existence of  
9 far older populations, such as those in the Japanese archipelago which are estimated to have  
10 colonised freshwater ~170,000 years ago (Kakioka et al. 2020), raises the question as to the  
11 fate of differential methylation over longer periods. Over time, for example, the initially  
12 heightened methylation variance may return to a less variable state due to refinement of  
13 methylation states via selection or the removal of the CpG sites via accumulation of C-T  
14 transitions. Alternatively, no substantial accumulation of mutations over time would suggest that  
15 the heightened diversity of FW-hypo sites reflects standing genetic variation.

16 If, indeed, heightened methylation variance arises due to relaxed control of methylation state,  
17 the mechanisms by which this could occur are not known. Artemov et al. (2017) suggested that  
18 mutations in genes encoding epigenetic regulators may underlie increased methylation  
19 variance, but did not identify any known epigenetic regulators in the vicinity of genomic regions  
20 differentiating marine and freshwater populations in the White Sea region. *Trans*- and *cis*-  
21 meQTL have however been identified in stickleback (Hu et al. 2021), some of which are indeed  
22 in the vicinity of genomic regions of high  $F_{st}$  between marine and freshwater populations.  
23 Differential selection on *trans*-meQTL in particular could have knock on effects on methylation  
24 sites across the genome.

25 While our study considered only genetic variation in the form of SNPs at CpG sites themselves,  
26 DNAm is associated with other types of genome sequence alterations. These include  
27 mutations in non-CpG context (Walser and Furano 2010), recombination rate variation (Mirouze  
28 et al. 2012), and structural variation including copy number and transposable element variation  
29 (Guerrero-Bosagna 2020). Indeed, the role of structural variation in local adaptation is  
30 increasingly appreciated and major inversions, transposable elements and copy number  
31 variants are all proposed to have played a role in stickleback freshwater adaptation (Reid et al.  
32 2021). The interplay between epigenetic variation and other forms of genetic variation therefore

1 warrants further interrogation in the local adaptation context (see however Kim et al. 2015;  
2 Huang and Chain 2021).

3 Finally, while shifts in nucleotide diversity in certain methylation contexts may signify changes in  
4 the fitness landscape of methylation sites, how they translate to fitness itself at the organism-  
5 level remains to be elucidated. Exploring the influence of methylation site diversity on gene  
6 expression variation would be a step towards addressing the possible fitness consequences.

## 7 *Conclusions*

8 By intersecting genetic and epigenetic data from naturally diverging populations, we have  
9 identified signatures of differential selection on DNAm sites which, combined with patterns of  
10 methylation variance and environmental inducibility, support a hypothesis that differential  
11 methylation is driven by shifts in the degree of intrinsic control of methylation state in a derived  
12 population. Shifts in this control seem to precede increases in nucleotide diversity and may  
13 therefore indicate parts of the genome where diversification is imminent. Heightened diversity of  
14 DMCs may also reflect a soft sweep which retains diversity at both genetic and epigenetic  
15 levels, a scenario compatible with previous genetic studies of local adaptation in stickleback  
16 (Terekhanova et al. 2014; Roberts Kingman et al. 2021). Indeed, our results support the idea  
17 that epigenetic variation should be incorporated alongside genetic mechanisms of adaptation in  
18 models of species adaptation and evolutionary potential (Bernatchez 2016). Our analyses  
19 demonstrate the exciting potential held in published datasets for exploring combined patterns of  
20 genome and epigenome evolution. Further investigation is now required to evaluate the broader  
21 role of methylome variation in shaping genomic landscapes across populations and species,  
22 and ultimately the influence of these interactions on fitness at the phenotypic level.

## 23 **Materials and Methods**

### 24 *Datasets*

25 We obtained a reduced-representation bisulfite sequencing (RRBS) dataset published by  
26 Artemov et al. (2017) (SRA project accession: PRJNA324599) comprising a total of six marine  
27 sticklebacks (of which three were exposed to lower salinity) and six freshwater sticklebacks (of  
28 which three were exposed to higher salinity; however, one of these three samples was excluded  
29 due to incomplete bisulfite conversion). The freshwater fish used for RRBS were sampled from  
30 Lake Mashinnoye, while the marine fish were sampled from the Kandalaksha gulf. Freshwater  
31 fish were also collected for pool-seq by Terekhanova et al. (2014) from Lake Mashinnoye (SRA

1 run accession: SRR869609), while marine fish were collected from the Kandalaksha gulf as part  
2 of the 2014 study and a subsequent 2019 study (Terekhanova et al. 2019). We selected the  
3 'White Sea, WSBS' sample from Terekhanova et al. (2019) (SRR7470095) as the marine  
4 sample for our comparison, given that it has a similar pool size to the Mashinnoye sample (12  
5 vs 10) and a similar number of 100bp paired reads (64,176,648 vs 62,016,859 after quality  
6 trimming). Sequence files were obtained in FASTQ format from the Sequence Read Archive  
7 (SRA) and European Nucleotide Archive (ENA).

#### 8 *Data processing: RRBS*

9 Raw RRBS reads were trimmed using TrimGalore v0.6.6 using default settings. Alignment to  
10 the Three-spined stickleback v.5 assembly (Nath et al. 2021) and subsequent methylation  
11 calling were carried out using Bismark v0.22.3 (Krueger and Andrews 2011) with Bowtie2  
12 v2.3.4.1 as the aligner (Langmead and Salzberg 2012). Methylation calls were not strand-  
13 specific. To remove sites harbouring C-T/G-A SNPs which otherwise contribute erroneous  
14 counts of non-methylated Cs, we ran three SNP-callers on each sample: BS-SNPper v1.1 (Gao  
15 et al. 2015), Biscuit v0.3.14 (<https://github.com/zhou-lab/biscuit>), and CGmap-tools v0.1.2 (Guo  
16 et al. 2018). We then compiled the coordinates of all sites harbouring C-T/G-A SNPs detected in  
17 any of the individuals by any of the SNP-callers (either homo- or heterozygous), and removed  
18 these sites from the Bismark coverage files containing the methylation counts (counts of Cs and  
19 Ts at each position). This approach detected 75% of C-T/G-A that were detected at high  
20 frequency in the freshwater pool-seq sample (Fig. S5). Further details of SNP calling from  
21 RRBS are provided in the supplementary methods.

#### 22 *Data processing: Pool-seq*

23 Raw reads were trimmed with Trimmomatic v0.36 (Bolger et al. 2014) with the option  
24 SLIDINGWINDOW:4:20 and otherwise default parameters. Only reads which remained paired  
25 after trimming were kept. Reads were mapped to the Three-spined stickleback v.5 assembly  
26 with Bowtie2 v2.3.4.1 with default parameters (Langmead and Salzberg 2012). Sambamba  
27 v0.7.1 (Tarasov et al. 2015) was used to filter the alignments to retain those with MAPQ  $\geq$ 20  
28 and to remove PCR duplicates. This resulted in 46,588,899 and 35,691,831 high quality  
29 alignments from marine and FW samples, equating to average genome coverage of 10.4x and  
30 8x, respectively. Samtools v0.1.18 (Danecek et al. 2021) was used to generate a pileup file from  
31 each BAM file, as required for the Popoolation and Popoolation2 toolkits.

#### 32 *Identification of differentially methylated CpG sites (DMCs) and subsampling of Non-DMCs*

1 Site-level differential methylation analysis was carried out using the methylKit R package  
2 v1.22.0 (Akalin et al. 2012), inputting the SNP-filtered Bismark coverage files. We omitted sites  
3 which did not have at least 5x coverage in each of the 11 samples as well as sites located on  
4 the mitochondrial chromosome and two sex chromosomes (chromosomes XIX and Y). We then  
5 filtered out sites that had either 0% or 100% methylation (i.e. no variation) in all samples from  
6 the main population comparison (3x marine and 3x freshwater). Three differential methylation  
7 analyses were then performed separately, comprising the comparisons also described in  
8 Artemov et al (2017): (1) marine fish in saltwater vs freshwater fish in freshwater (main  
9 population comparison), (2) marine fish in saltwater vs marine fish in freshwater (marine low  
10 salinity treatment), and (3) freshwater fish in freshwater vs freshwater fish in saltwater  
11 (freshwater high salinity treatment). All groups comprised N=3 with the exception of freshwater  
12 fish in saltwater (N=2, due to low bisulfite conversion efficiency of sample SRR3632642).  
13 Regardless, we considered sites to be differentially methylated given a difference in percentage  
14 methylation of  $\geq 15$  and a FDR-corrected p-value of  $\leq 0.05$ . The purpose of the experimental  
15 comparisons was to identify which population-DMCs were also induced by salinity change, and  
16 so we did not consider sites that were induced by salinity change but not differentially  
17 methylated between populations. We also did not consider the direction of induced change  
18 (hypo- or hypermethylated in response to salinity change). Subsequently, we detected 91,320  
19 sites that were hypomethylated in freshwater compared to marine (of which 10,289 were  
20 induced in either population) and 14,508 sites that were hypermethylated in freshwater  
21 compared to marine (of which 5685 were induced in either population). 895,121 sites were not  
22 differentially methylated between populations, a subsample of which we would use as reference  
23 sites when examining nucleotide diversity. Due to the possibility that DMCs could be distributed  
24 non-randomly across a chromosome and given that nucleotide diversity can vary across a  
25 chromosome (e.g. lower diversity in centromeric regions), we used a sampling procedure which  
26 randomly selected one non-differentially methylated site that was within 2kb upstream or 2kb  
27 downstream of each DMC. After removing duplicate samples, this resulted in a subsample of  
28 99,585 non-differentially methylated CpG sites (Non-DMCs).

### 29 *Nucleotide diversity of site categories*

30 We used the variance-at-position.pl script from the Popoolation toolkit (Kofler, Orozco-  
31 terWengel, et al. 2011) to calculate within-population nucleotide diversity statistics ( $\pi$ ,  
32 Watterson's  $\theta$ , and Tajima's  $D$ ) for different categories of site (e.g. Non-DMC, FW-hypo, FW-  
33 hyper) on each chromosome for each population. The decision to obtain estimates of  $\pi$  on a

1 per-chromosome basis was because Popoolation's estimates of  $\pi$  are accurate over large  
2 numbers of sites, but not at the single site level (Kofler, Orozco-terWengel, et al. 2011). Each  
3 site was labelled with its chromosome and its category within the analysis (e.g. chr1 FW-hypo)  
4 and the labelled category was entered as the 'gene ID' in a GTF file, such that variance-at-  
5 position.pl, which was developed to calculate diversity statistics per-gene, was instructed to  
6 calculate  $\pi$  for each combination of chromosome and site category. A similar procedure was  
7 used to obtain  $\pi$  for sites ranked according to (difference in) mean percentage methylation  
8 (Mean{PM}), (difference in) SD of percentage methylation ( $SD_{meth}$ ), and absolute inducibility,  
9 whereby ranks were assigned using the bin() function from the OneR package, specifying 50  
10 ranks each time. Sites were then labelled in the GTF according to their rank (regardless of  
11 chromosome), such that a single value of  $\pi$  was obtained for each rank. Variance-at-position.pl  
12 from Popoolation was run with the parameters --min-qual 20 --min-coverage 3 --min-count 2.  
13 The majority of sites met the requisite 3x coverage for inclusion in nucleotide diversity estimates  
14 of marine (99%) and FW (93%). For the analysis of nucleotide diversity as a function of absolute  
15 inducibility, one rank was excluded from the FW population due to insufficient coverage (<60%  
16 of sites with 3x coverage).

17 Fst for different categories of methylation sites (including ranked sites) were obtained using the  
18 Popoolation2 toolkit (Kofler, Pandey, et al. 2011). 'Gene-wise' .sync files were obtained from the  
19 pileup files using coordinates in the abovementioned gtf files and were used as input for the 'fst-  
20 sliding.pl' script which was run with parameters --min-count 2 --min-coverage 3 --pool-size 22 --  
21 min-covered-fraction 0 --max-coverage 1000 --window-size 1000000 --step-size 1000000.

### 22 *Percentage of sites with SNPs*

23 To obtain the % of sites within each result category (Non-DMC, FW-hypo, and FW-hyper)  
24 harbouring SNPs of different types (C-T/G-A and non-C-T/G-A) in the pool-seq samples, we  
25 filtered the BAM files of each population to retain alignments corresponding with the positions of  
26 interest. We then ran GATK HaplotypeCaller (McKenna et al. 2010) with the --sample-ploidy set  
27 to the pool size x 2 (24 for marine and 20 for freshwater), and otherwise default settings. The  
28 subsequent VCF file was then filtered using bcftools v1.10 to retain only biallelic SNPs at the  
29 sites of interest. We subsequently extracted from the VCF a list of reference and alternate  
30 alleles at sites of interest harbouring biallelic SNPs. We were therefore able to assign SNPs as  
31 either 'C-T/G-A' or 'other', and calculate the % of sites in each result category harbouring  
32 biallelic SNPs of one of those two classes.



- 1 Barrett RDH, Rogers SM, Schluter D. 2008. Natural selection on a major armor gene in threespine  
2 stickleback. *Science* 322:255–257.
- 3 Bernatchez L. 2016. On the maintenance of genetic variation and adaptation to environmental change:  
4 considerations from population genomics in fishes. *J Fish Biol* 89:2519–2556.
- 5 Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data.  
6 *Bioinformatics* 30:2114–2120.
- 7 Danecek P, Bonfield JK, Liddle J, Marshall J, Ohan V, Pollard MO, Whitwham A, Keane T, McCarthy SA,  
8 Davies RM, et al. 2021. Twelve years of SAMtools and BCFtools. *Gigascience* 10:1–4.
- 9 Day T, Pritchard J, Schluter D. 1994. A COMPARISON OF TWO STICKLEBACKS. *Evolution* 48:1723–  
10 1734.
- 11 Dubin MJ, Zhang P, Meng D, Remigereau MS, Osborne EJ, Casale FP, Drewe P, Kahles A, Jean G,  
12 Vilhjálmsson B, et al. 2015. DNA methylation in Arabidopsis has a genetic basis and shows evidence of  
13 local adaptation. *Elife* 4:e05255.
- 14 Flores KB, Wolschin F, Amdam GV 2013. The Role of Methylation of DNA in Environmental Adaptation.  
15 *Integr Comp Biol* 53:359.
- 16 Gao S, Zou D, Mao L, Liu H, Song P, Chen Y, Zhao S, Gao C, Li X, Gao Z, et al. 2015. BS-SNPer: SNP  
17 calling in bisulfite-seq data. *Bioinformatics* 31:4006–4008.
- 18 Guerrero-Bosagna C. 2012. Finalism in Darwinian and Lamarckian Evolution: Lessons from Epigenetics  
19 and Developmental Biology. *Evol Biol* 39:283–300.
- 20 Guerrero-Bosagna C. 2020. From epigenotype to new genotypes: Relevance of epigenetic mechanisms  
21 in the emergence of genomic evolutionary novelty. *Semin Cell Dev Biol* 97:86–92.
- 22 Guo W, Zhu P, Pellegrini M, Zhang MQ, Wang X, Ni Z. 2018. CGmapTools improves the precision of  
23 heterozygous SNV calls and supports allele-specific methylation detection and visualization in bisulfite-  
24 sequencing data. *Bioinformatics* 34:381–387.
- 25 Hasan MM, DeFaveri J, Kuure S, Dash SN, Lehtonen S, Merilä J, McCairns RJS. 2017. Sticklebacks  
26 adapted to divergent osmotic environments show differences in plasticity for kidney morphology and  
27 candidate gene expression. *J Exp Biol* 220:2175–2186.
- 28 Heckwolf MJ, Meyer BS, Häslér R, Höppner MP, Eizaguirre C, Reusch TBH. 2020. Two different  
29 epigenetic information channels in wild three-spined sticklebacks are involved in salinity adaptation. *Sci*  
30 *Adv* 6:eaaz1138.
- 31 Hermisson J, Pennings PS. 2017. Soft sweeps and beyond: understanding the patterns and probabilities  
32 of selection footprints under rapid adaptation. *Methods Ecol Evol* 8:700–716.
- 33 He X-J, Chen T, Zhu J-K. 2011. Regulation and function of DNA methylation in plants and animals. *Cell*  
34 *Res* 21:442–465.
- 35 Huang KM, Chain FJJ. 2021. Copy number variations and young duplicate genes have high methylation  
36 levels in sticklebacks. *Evolution* 75:706–718.
- 37 Hu J, Wuitchik SJS, Barry TN, Jamniczky HA, Rogers SM, Barrett RDH. 2021. Heritability of DNA  
38 methylation in threespine stickleback (*Gasterosteus aculeatus*). *Genetics* 217:1-15.
- 39 Jackson BC, Campos JL, Zeng K. 2015. The effects of purifying selection on patterns of genetic  
40 differentiation between *Drosophila melanogaster* populations. *Heredity* 114:163.
- 41 Johnson KM, Kelly MW. 2020. Population epigenetic divergence exceeds genetic divergence in the  
42 Eastern oyster *Crassostrea virginica* in the Northern Gulf of Mexico. *Evol Appl* 13:945–959.
- 43 Johnson LJ, Tricker PJ. 2010. Epigenomic plasticity within populations: its evolutionary significance and  
44 potential. *Heredity* 105:113–121.
- 45 Jones FC, Grabherr MG, Chan YF, Russell P, Mauceli E, Johnson J, Swofford R, Pirun M, Zody MC,  
46 White S, et al. 2012. The genomic basis of adaptive evolution in threespine sticklebacks. *Nature* 484:55–  
47 61.

- 1 Kakioka R, Mori S, Kokita T, Hosoki TK, Nagano AJ, Ishikawa A, Kume M, Toyoda A, Kitano J. 2020.  
2 Multiple waves of freshwater colonization of the three-spined stickleback in the Japanese Archipelago.  
3 *BMC Evol Biol* 20:143.
- 4 Kilvitis HJ, Hanson H, Schrey AW, Martin LB. 2017. Epigenetic Potential as a Mechanism of Phenotypic  
5 Plasticity in Vertebrate Range Expansions. *Integr Comp Biol* 57:385–395.
- 6 Kim K do, el Baidouri M, Abernathy B, Iwata-Otsubo A, Chavarro C, Gonzales M, Libault M, Grimwood J,  
7 Jackson SA. 2015. A Comparative Epigenomic Analysis of Polyploidy-Derived Genes in Soybean and  
8 Common Bean. *Plant Physiol* 168:1433–1447.
- 9 Kirch M, Romundset A, Gilbert MTP, Jones FC, Foote AD. 2021. Ancient and modern stickleback  
10 genomes reveal the demographic constraints on adaptation. *Curr Biol* 31:2027–2036.
- 11 Klughammer J, Romanovskaia D, Nemc A, Posautz A, Seid CA, Schuster LC, Keinath MC, Lugo Ramos  
12 JS, Kosack L, Evankow A, et al. 2023. Comparative analysis of genome-scale, base-resolution DNA  
13 methylation profiles across 580 animal species. *Nat Commun* 14:232.
- 14 Kofler R, Orozco-terWengel P, de Maio N, Pandey RV, Nolte V, Futschik A, Kosiol C, Schlötterer C. 2011.  
15 PoPoolation: A Toolbox for Population Genetic Analysis of Next Generation Sequencing Data from  
16 Pooled Individuals. *PLoS One* 6:e15925.
- 17 Kofler R, Pandey RV, Schlötterer C. 2011. PoPoolation2: identifying differentiation between populations  
18 using sequencing of pooled DNA samples (Pool-Seq). *Bioinformatics* 27:3435–3436.
- 19 Krueger F, Andrews SR. 2011. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq  
20 applications. *Bioinformatics* 27:1571–1572.
- 21 Lämke J, Bäurle I. 2017. Epigenetic and chromatin-based mechanisms in environmental stress  
22 adaptation and stress memory in plants. *Genome Biol* 18:124.
- 23 Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. *Nature Methods* 9:357–359.
- 24 Lescak EA, Bassham SL, Catchen J, Gelmond O, Sherbick ML, van Hippel FA, Cresko WA. 2015.  
25 Evolution of stickleback in 50 years on earthquake-uplifted islands. *Proc Natl Acad Sci USA* 112:E7204–  
26 E7212.
- 27 Lindner M, Gawehns F, te Molder S, Visser ME, van Oers K, Laine VN. 2022. Performance of methods to  
28 detect genetic variants from bisulphite sequencing data in a non-model species. *Mol Ecol Resour* 22:834–  
29 846.
- 30 Loyfer N, Magenheimer J, Peretz A, Cann G, Bredno J, Klochendler A, Fox-Fisher I, Shabi-Porat S, Hecht  
31 M, Pelet T, et al. 2023. A DNA methylation atlas of normal human cell types. *Nature* 613:355–364.
- 32 Lu Z, Cui J, Wang L, Teng N, Zhang S, Lam HM, Zhu Y, Xiao S, Ke W, Lin J, et al. 2021. Genome-wide  
33 DNA mutations in *Arabidopsis* plants after multigenerational exposure to high temperatures. *Genome Biol*  
34 22:1–27.
- 35 McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytzky A, Garimella K, Altshuler D,  
36 Gabriel S, Daly M, et al. 2010. The Genome Analysis Toolkit: A MapReduce framework for analyzing  
37 next-generation DNA sequencing data. *Genome Res* 20:1297–1303.
- 38 Mirouze M, Lieberman-Lazarovich M, Aversano R, Bucher E, Nicolet J, Reinders J, Paszkowski J. 2012.  
39 Loss of DNA methylation affects the recombination landscape in *Arabidopsis*. *Proc Natl Acad Sci USA*  
40 109:5880–5885.
- 41 Nath S, Shaw DE, White MA. 2021. Improved contiguity of the threespine stickleback genome using long-  
42 read sequencing. *G3* 11: jkab007.
- 43 Nei M, Li WH. 1979. Mathematical model for studying genetic variation in terms of restriction  
44 endonucleases. *Proc Natl Acad Sci USA* 76:5269–5273.
- 45 Pan W, Godoy RS, Cook DP, Scott AL, Nurse CA, Jonz MG. 2022. Single-cell transcriptomic analysis of  
46 neuroepithelial cells and other cell types of the gills of zebrafish (*Danio rerio*) exposed to hypoxia. *Sci Rep*  
47 12:1–17.



- 1 Poulos RC, Olivier J, Wong JWH. 2017. The interaction between cytosine methylation and processes of  
2 DNA replication and repair shape the mutational landscape of cancer genomes. *Nucleic Acids Res*  
3 45:7786–7795.
- 4 Rastorguev SM, Nedoluzhko A v., Gruzdeva NM, Boulygina ES, Sharko FS, Ibragimova AS, Tsygankova  
5 S v., Artemov A v., Skryabin KG, Prokhortchouk EB. 2017. Differential miRNA expression in the three-  
6 spined stickleback, response to environmental changes. *Sci Rep* 7:18089.
- 7 Rastorguev SM, Nedoluzhko A v., Sharko FS, Boulygina ES, Sokolov AS, Gruzdeva NM, Skryabin KG,  
8 Prokhortchouk EB. 2016. Identification of novel microRNA genes in freshwater and marine ecotypes of  
9 the three-spined stickleback (*Gasterosteus aculeatus*). *Mol Ecol Resour* 16:1491–1498.
- 10 R Development Core Team. 2011. R: A Language and Environment for Statistical Computing. Vienna,  
11 Austria: R Foundation for Statistical Computing Available from: <http://www.r-project.org>
- 12 Reid K, Bell MA, Veeramah KR. 2021. Threespine Stickleback: A Model System For Evolutionary  
13 Genomics. *Annu Rev Genomics Hum Genet* 22:357.
- 14 Richards EJ. 2006. Inherited epigenetic variation — revisiting soft inheritance. *Nature Reviews Genetics*  
15 7:395–401.
- 16 Roberts Kingman GA, Vyas DN, Jones FC, Brady SD, Chen HI, Reid K, Milhaven M, Bertino TS, Aguirre  
17 WE, Heins DC, et al. 2021. Predicting future from past: The genomic basis of recurrent and rapid  
18 stickleback evolution. *Sci Adv* 7:eabg5285.
- 19 Sammarco I, Münzbergová Z, Latzel V. 2022. DNA Methylation Can Mediate Local Adaptation and  
20 Response to Climate Change in the Clonal Plant *Fragaria vesca*: Evidence From a European-Scale  
21 Reciprocal Transplant Experiment. *Front Plant Sci* 13:435.
- 22 Shirai K, Sato MP, Nishi R, Seki M, Suzuki Y, Hanada K. 2021. Positive selective sweeps of epigenetic  
23 mutations regulating specialized metabolites in plants. *Genome Res* 31:1060–1068.
- 24 Smith G, Smith C, Kenny JG, Chaudhuri RR, Ritchie MG. 2015. Genome-Wide DNA Methylation Patterns  
25 in Wild Samples of Two Morphotypes of Threespine Stickleback (*Gasterosteus aculeatus*). *Mol Biol Evol*  
26 32:888–895.
- 27 Smith TA, Martin MD, Nguyen M, Mendelson TC. 2016. Epigenetic divergence as a potential first step in  
28 darter speciation. *Mol Ecol* 25:1883–1894.
- 29 Stajic D, Perfeito L, Jansen LET. 2019. Epigenetic gene silencing alters the mechanisms and rate of  
30 evolutionary adaptation. *Nat Ecol Evol* 3:491-498.
- 31 Stajich JE, Hahn MW. 2005. Disentangling the Effects of Demography and Selection in Human History.  
32 *Mol Biol Evol* 22:63–73.
- 33 Storz JF, Natarajan C, Signore AV, Witt CC, McCandlish DM, Stoltzfus A. 2019. The role of mutation bias  
34 in adaptive molecular evolution: insights from convergent changes in protein function. *Philo Trans Royal*  
35 *Soc Lond B Biol Sci* 374:20180238.
- 36 Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism.  
37 *Genetics* 123:585–595.
- 38 Tarasov A, Vilella AJ, Cuppen E, Nijman IJ, Prins P. 2015. Sambamba: fast processing of NGS alignment  
39 formats. *Bioinformatics* 31:2032–2034.
- 40 Terekhanova NV, Barmintseva AE, Kondrashov AS, Bazykin GA, Mugue NS, Alba M. 2019. Architecture  
41 of Parallel Adaptation in Ten Lacustrine Threespine Stickleback Populations from the White Sea Area.  
42 *Genome Biol Evol* 11:2605–2618.
- 43 Terekhanova NV, Logacheva MD, Penin AA, Neretina T v, Barmintseva AE. 2014. Fast Evolution from  
44 Precast Bricks: Genomics of Young Freshwater Populations of Threespine Stickleback *Gasterosteus*  
45 *aculeatus*. *PLoS Genet* 10:1004696.
- 46 Vernaz G, Malinsky M, Svoldal H, Du M, Tyers AM, Santos ME, Durbin R, Genner MJ, Turner GF, Miska  
47 EA. 2021. Mapping epigenetic divergence in the massive radiation of Lake Malawi cichlid fishes. *Nat*  
48 *Commun* 12:5870.

- 1 Villicaña S, Bell JT. 2021. Genetic impacts on DNA methylation: research findings and future  
2 perspectives. *Genome Biol* 22:127.
- 3 Walser J-C, Furano AV. 2010. The mutational spectrum of non-CpG DNA varies with CpG content.  
4 *Genome Res* 20:875–882.
- 5 Weir BS, Cockerham CC. 1984. Estimating F-Statistics for the Analysis of Population Structure. *Evolution*  
6 38:1358.
- 7 Wickham H. 2011. ggplot2. *Wiley Interdiscip Rev Comput Stat* 3:180–185.
- 8 Wulfridge P, Langmead B, Feinberg AP, Hansen KD. 2019. Analyzing whole genome bisulfite sequencing  
9 data from highly divergent genotypes. *Nucleic Acids Res* 47:117.
- 10 Xia J, Han L, Zhao Z. 2012. Investigating the relationship of DNA methylation with mutation rate and  
11 allele frequency in the human genome. *BMC Genomics* 13 Suppl 8:S7.
- 12 Zhou Y, He F, Pu W, Gu X, Wang J, Su Z. 2020. The Impact of DNA Methylation Dynamics on the  
13 Mutation Rate During Human Germline Development. *G3* 10:3337–3346.

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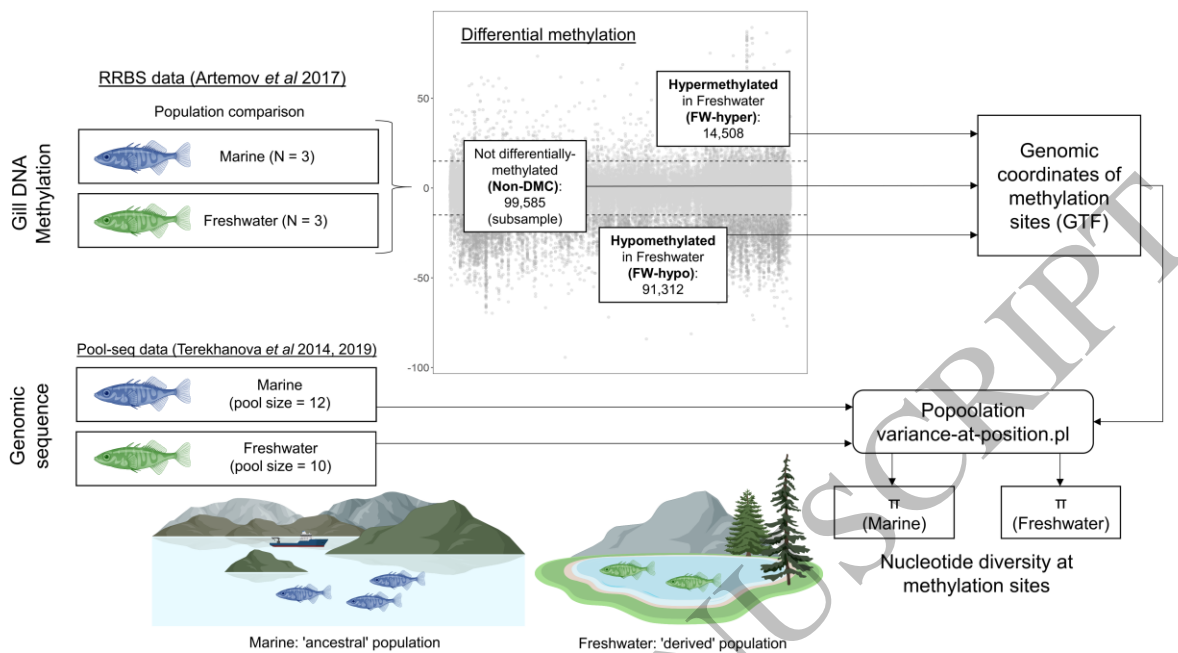
15 **Figure 1. Analysis workflow for obtaining nucleotide diversity estimates for differentially**  
16 **methyated sites.** Site-level differential methylation analysis was performed to compare marine  
17 (considered as ‘ancestral’ population) vs freshwater sticklebacks (considered as ‘derived’  
18 population) using gill RRBS data previously published by Artemov et al. (2017). Taking the  
19 marine population as the reference methylation state, sites were classified as not differentially-  
20 methylated (Non-DMC; no significant difference in percentage of methylated copies between  
21 populations), FW-hypo (significantly lower percentage of methylated copies in freshwater  
22 compared to marine), or FW-hyper (significantly higher percentage of methylated copies in  
23 freshwater compared to marine). For Non-DMCs, a subset of the total was used, comprising  
24 ~11% of the total Non-DMCs (see methods). Coordinates of sites belonging to the three site  
25 classes (Non-DMC, FW-hypo, and FW-hyper) were compiled in a GTF file for use with the  
26 variance-at-position.pl script from the Popoolation toolkit. The nucleotide diversity ( $\pi$ ) of each  
27 site class on each chromosome was estimated from whole-genome pool-seq data  
28 (Terekhanova et al. 2014, 2019) of marine and freshwater fish derived from the same or similar  
29 geographic locations as those taken for the RRBS data.

30 **Figure 2. Nucleotide diversity of differentially methylated sites. (A)**  $\pi$  (average number of  
31 pairwise differences), **(B)**  $F_{st}$  (marine vs. freshwater), and **(C)** Tajima’s  $D$  estimated from pool-  
32 seq of marine and freshwater sticklebacks for three classes of methylation site identified from  
33 RRBS individuals and classified according to the direction of methylation difference in  
34 freshwater fish compared to marine (Not differentially-methylated (Non-DMC), hypomethylated  
35 in freshwater (FW-hypo), or hypermethylated in freshwater (FW-Hyper)). **(D)** Percentage of sites  
36 in each site class harbouring biallelic SNPs of the type C-T/G-A (upper panel) or other types (C-  
37 A/G-T or C-G/G-T, lower panel), estimated separately for each chromosome. Average numbers



1 **Fig. 5. Nucleotide diversity of differentially methylated sites in relation to their capacity**  
2 **for induced methylation change in response to environmental salinity. (A)** Additional  
3 RRBS data deriving from experimental salinity treatments performed by Artemov et al (2017)  
4 (marine fish placed in freshwater and freshwater fish placed in saltwater) were used to identify  
5 sites that were inducible in response to salinity change in either of the two populations. **(B)** % of  
6 sites in the Non-DMC, FW-hypo, and FW-hyper categories that were induced in response to  
7 salinity change in the marine (blue) and freshwater (green) populations. *P*-values derived from  
8 paired Wilcoxon tests. **(C)** Per-chromosome estimates of  $\pi$  for FW-hypo and FW-hyper sites  
9 divided according their capacity for induced gill methylation change in response to a change in  
10 environmental salinity, considering sites that were induced in neither of the populations, either of  
11 the two populations, or only in one of the two populations (marine or freshwater).  $\pi$  of Non-  
12 DMCs is shown in separate panel for comparison. *P*-values derived from paired *t*-tests. **(D)**  $\pi$  of  
13 inducible DMCs that were ranked according to their mean absolute induced change in  
14 percentage of methylation (i.e. regardless of the direction). Within each population, only sites  
15 that were significantly differentially methylated in response to salinity (mean difference in  
16 percentage of methylation  $\geq 15$  or  $\leq -15$ ,  $p < 0.05$ ) were considered. Separate ranks were  
17 obtained for marine and FW and a single  $\pi$  estimate obtained for each rank. **(E)** Per-  
18 chromosome estimates of pairwise *F*<sub>st</sub> (freshwater vs. marine) of FW-hypo and FW-hyper sites  
19 divided according to their capacity for induced methylation change. *F*<sub>st</sub> of Non-DMCs is shown  
20 in separate panel for comparison. *P*-values derived from paired *t*-tests. **(F)** Pairwise *F*<sub>st</sub> of  
21 inducible DMCs that were ranked according to their mean absolute induced change in  
22 percentage of methylation (i.e. regardless of the direction of the change). For **(D)** & **(F)**, 50 ranks  
23 were used for marine and 49 for freshwater. Each rank contains an average of 227 sites for  
24 Marine and 228 sites for Freshwater. Trend lines derived from linear models and ribbons show  
25 SEM.

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**Figure 1**  
159x91 mm (.97 x DPI)

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ACCEPTED MANUSCRIPT

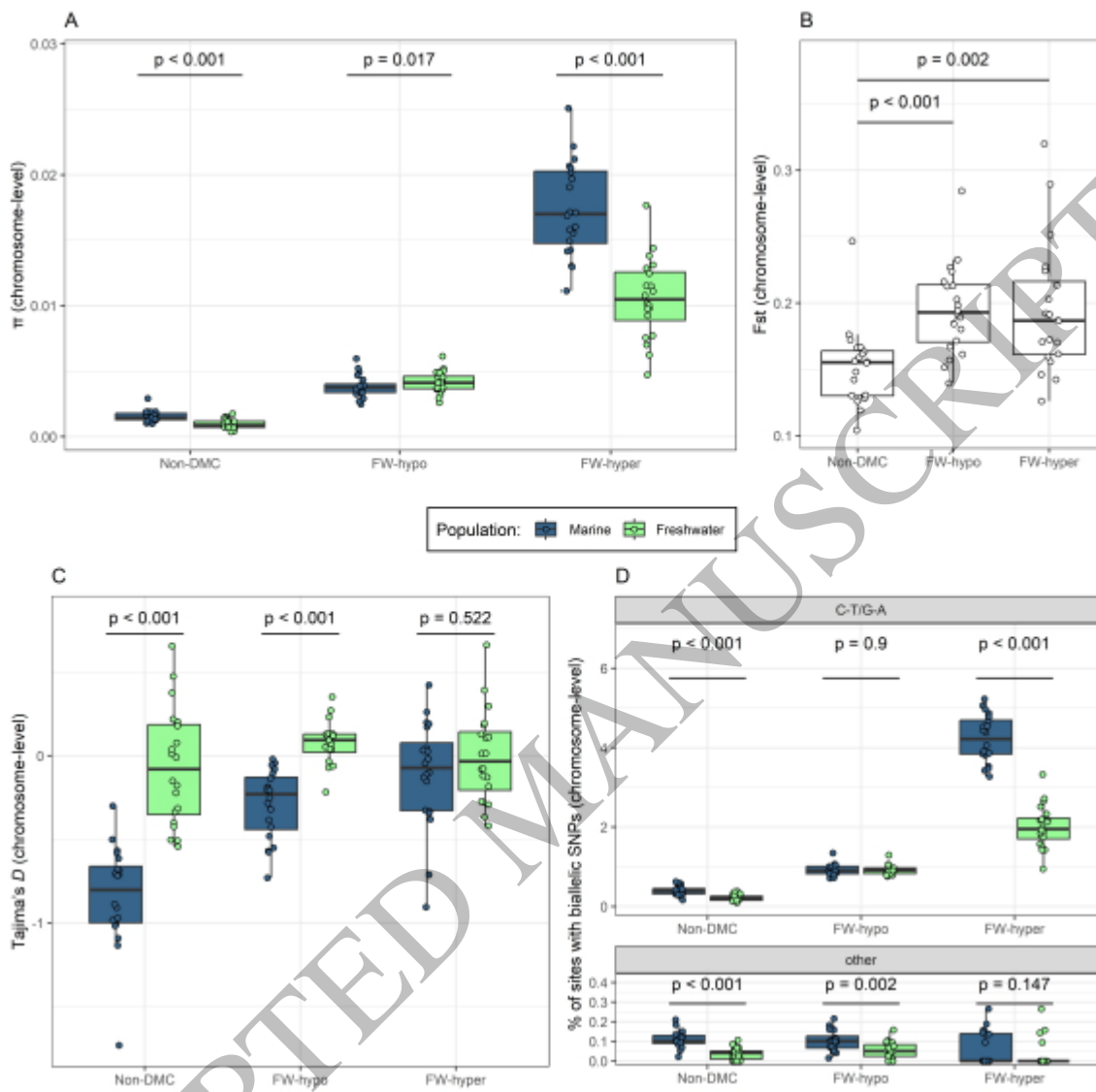
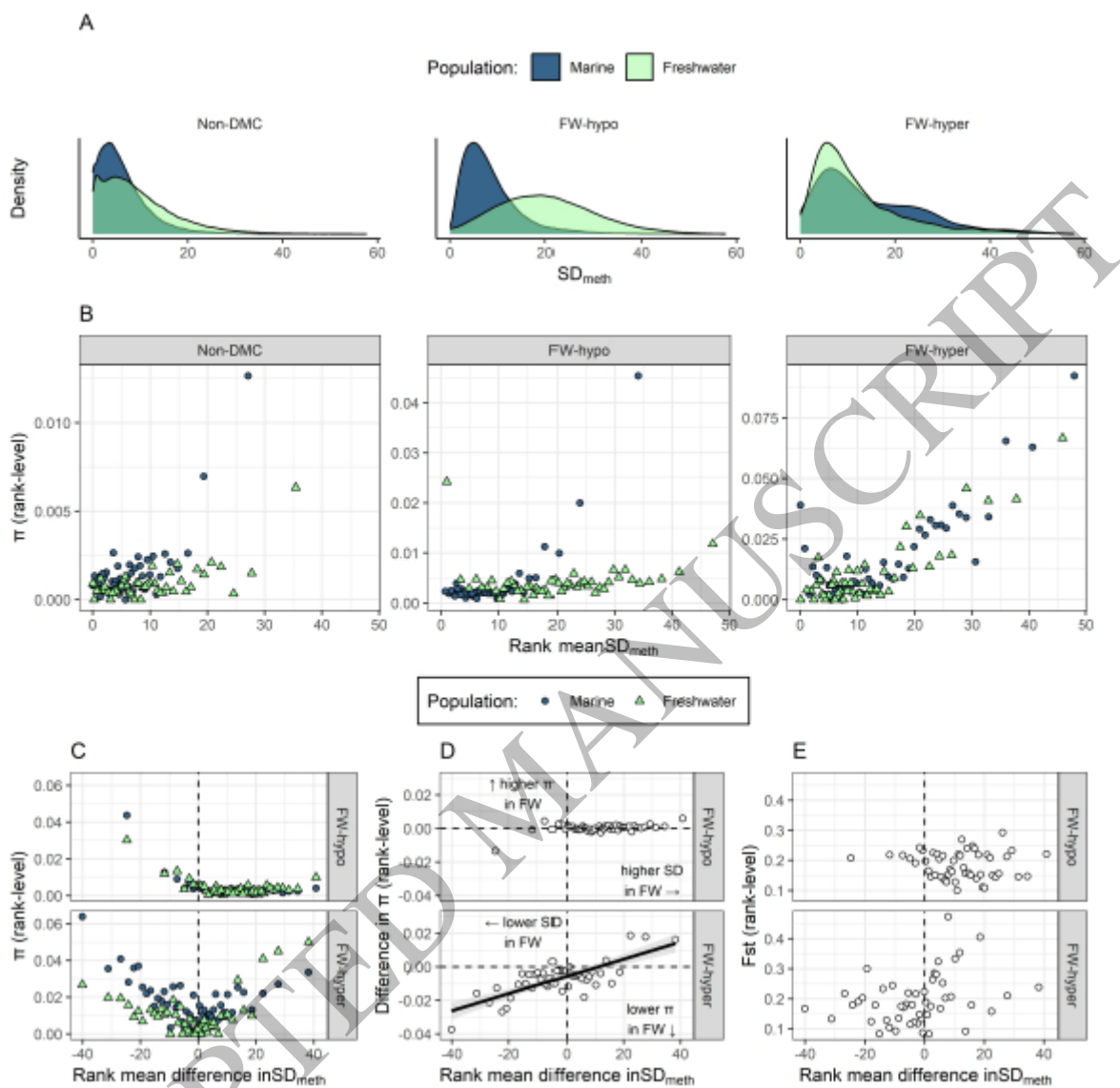


Figure 2  
153x150 mm (.97 x DPI)

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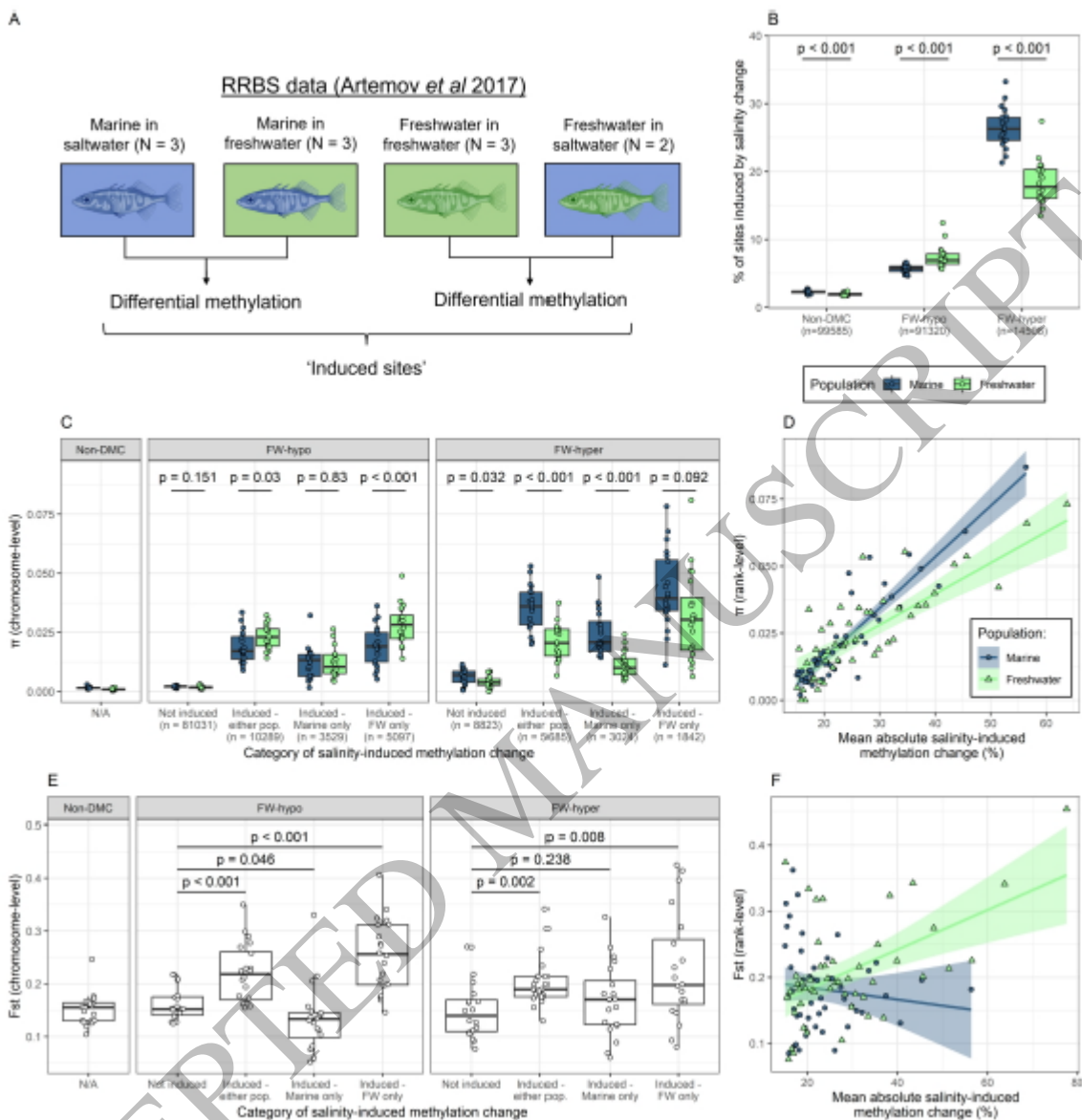




**Figure 4**  
152x152 mm (.97 x DPI)

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**Figure 5**  
152x156 mm (.97 x DPI)

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