DNA methylation facilitates local adaptation and adaptive transgenerational plasticity.

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Abstract
Epigenetic inheritance has been suggested to contribute to adaptation via two distinct pathways. Either, stable epigenetic marks emerge as epimutations and are targets of natural selection via the phenotype, analogous to adaptation from DNA sequence-based variation. Alternatively, epigenetic marks are inducible by environmental cues, possibly representing one mechanism of transgenerational phenotypic plasticity. We investigated whether both postulated pathways are detectable in nature and sequenced methylomes and genomes of three-spined sticklebacks.
(Gasterosteus aculeatus) across a natural salinity gradient in a space-for-time approach. Consistent with local adaptation patterns, stickleback populations showed differentially methylated CpG sites (pop-DMS) at genes enriched for osmoregulatory processes. In a two-generation salinity acclimation experiment with fish from the mid salinity, we found the majority (62%) of pop-DMS to be insensitive to experimental salinity change, suggesting that they were shaped by selection and facilitate local salinity adaptation. Among the experimentally inducible DMS, two-thirds increased in similarity to anticipated adaptive patterns in wild populations under exposure to the novel salinity. This study demonstrates the presence of two types of methylation marks, inducible and stable, that contribute to adaptive transgenerational plasticity and local adaptation in natural populations.

Main text

Recent advances in epigenetics have started to challenge our understanding of inheritance and adaptive evolution\(^1\).\(^2\).\(^3\). It has been suggested that epigenetic inheritance provides an additional evolutionary pathway to adaptive phenotypes\(^4\).\(^5\), involving the heredity of molecular variation such as DNA methylation, histone modification and small RNAs\(^6\). Several theoretical models posit that heritable variation of these molecular modifications can contribute to adaptation to environmental change via two distinct information pathways\(^5\).\(^7\).\(^8\). Firstly, selection-based epigenetic marks can emerge as spontaneous epimutations that remain stable across generations, regardless of the current environment\(^7\). These epimutations underlie phenotypes that are targets of natural selection similar to adaptation from DNA sequence-based variation\(^5\).\(^8\). Secondly, detection-based effects describe inducible epigenetic marks under environmental control and are hypothesized to be a transgenerational form of phenotypic plasticity\(^7\).\(^10\). While the significance and principal difference of both transmission pathways have been acknowledged\(^4\).\(^5\).\(^7\), empirical evidence for the quantification of adaptive epigenetic variation along with its transgenerational inducibility is rare, especially in natural populations (but see
One prime objective of this study was thus to assess whether these two epigenetic pathways can be detected in nature and to test whether short term acclimation responses match patterns of DNA methylation variation of natural populations. If transgenerational experiments result in DNA methylation profiles closer to those of locally adapted natural populations, this would provide evidence that DNA methylation is mechanistically involved in adaptive transgenerational plasticity.

Studying the adaptation to ocean salinity is particularly well suited to identify selection-based and detection-based effects, as changes in salinity are predicted to be gradual over time and experiments can easily be carried out with average values, as ocean salinity varies less depending on the season than temperature for instance. Since salinity change imposes strong physiological stress with well-defined cellular effects, natural salinity gradients offer unparalleled opportunities to use local patterns of epigenetic variation as background against which direction and magnitude of experimental salinity manipulations can be tested. One suitable ecosystem to follow such a space-for-time approach is the Baltic Sea, a semi-enclosed marginal sea that has been dubbed a time machine for many predicted perturbations associated with global change.

Taking advantage of this natural large-scale salinity gradient, we sequenced the methylomes (reduced representation bisulfite sequencing, RRBS) as well as whole genomes of three-spined sticklebacks (Gasterosteus aculeatus) from three locally adapted populations in- and outside the Baltic Sea salinity gradient (6, 20 and 33 PSU). Baltic stickleback populations are genetically differentiated from one another (genome wide average pairwise F\textsubscript{ST} = 0.028) and show patterns consistent with local adaptation to salinity regimes in controlled common garden experiments. Furthermore, sticklebacks are known for their adaptive transgenerational plasticity in response to variation in temperature and changes in DNA methylation levels at osmoregulatory genes in response to within generation salinity acclimation. However, it remains unclear whether DNA methylation facilitates adaptive transgenerational plasticity, a mechanism hypothesized to facilitate phenotypic adaptation to
rapid environmental change. To address this question, we complemented our field survey with a two-generation salinity acclimation experiment using the mid salinity population (20 PSU) to quantify the proportion of stable (potentially selection-based) and inducible (potentially detection-based) DNA methylation within- and across generations (Figure 1). We focused on the methylation of cytosines at cytosine-phosphate-guanine dinucleotides (CpG sites), the most common methylation motif in vertebrates\(^2\) with partial inheritance potentially facilitating adaptation in natural populations\(^10\).

We tested three hypotheses: (i) Stickleback populations originating from different salinity areas (6, 20 and 33 PSU) show differentially methylated CpG sites (pop-DMS), consistent with patterns of local adaptation. (ii) Such pop-DMS include experimentally stable, in the form of population-specific, and experimentally inducible, in the form of environment-specific, methylation sites. (iii) Upon transgenerational salinity acclimation, inducible DNA methylations become more similar to the patterns of natural populations at corresponding salinities. Along with the functional enrichment assessment, the latter findings would be evidence of a mechanism of adaptive transgenerational plasticity.

**Figure 1:** Experimental space-for-time approach. We characterized DNA methylation profiles (via Reduced Representation Bisulfite Sequencing, RRBS) and whole genomes (Whole Genome Sequencing,
WGS) of fish from three populations of wild caught three-spined sticklebacks locally adapted to 6 (blue, N = 15), 20 (green, N = 16) and 33 PSU (yellow, N = 15). We also collected sticklebacks from the mid salinity location (20 PSU) and acclimated laboratory bred offspring of these fish within one (‘within-generational’) or over two (‘transgenerational’) generations to decreased (6 PSU) or increased (33 PSU) salinity, and maintained a control group at its original salinity (N = 11-12 per group, see details in Figure). Differential methylation within and across generations was assessed and compared to natural populations locally adapted to the corresponding salinity, serving as the hypothetical future DNA methylation state to capture long term adaptation processes.

**Identifying differentially methylated CpG sites across wild populations along a salinity cline**

In order to identify differentially methylated CpG sites (DMS) between stickleback populations along the salinity cline in- and outside of the Baltic Sea (hereafter pop-DMS), we sequenced the methylome (RRBS) of 46 wild caught sticklebacks from 3 different salinity regimes (Sylt, 33 PSU; Kiel, 20 PSU; Nynäshamn, 6 PSU; Figure 1). After quality and coverage filtering, we obtained 525,985 CpG sites present in all groups, corresponding to ~4% of all CpG sites in the stickleback genome. Between pairs of wild caught populations, we detected 1,470 (comparison 20 vs. 6 PSU) and 1,158 (20 vs. 33 PSU) pop-DMS. The distribution of these sites was random with regard to the genomic features (promoter, exon, intron, and intergenic; 20 vs. 6 PSU: \(X^2_3 = 3.36, P = 0.340\); 20 vs. 33 PSU: \(X^2_3 = 1.61, P = 0.656\); Supplementary Material: Figure S1 and Table S1) and regions along the chromosomes (Supplementary Figure S3A). Noteworthy, among these pop-DMS, 1,098 (20 vs. 6 PSU) and 871 (20 vs. 33 PSU) were located close to (<10 kb from transcription start sites) or within genes thereby associated with 655 and 510 genes, respectively. Many of these genes were involved in basic biological processes such as ectoderm development, DNA-repair and strand renaturation, as well as chromatid segregation and chromosome condensation (Figure 2). Particularly relevant and concordant with previous
findings of local salinity adaptation\textsuperscript{15}, these genes were enriched for osmoregulatory processes such as ion transport and channel activity, renal water homeostasis and absorption. Genes associated with ≥ 10 pop-DMS are listed in Table 1 (for all genes, see Supplementary Table S2A and S2B). Since local adaptation is 10-fold more likely to involve changes at the gene expression than at the amino acid sequence level\textsuperscript{22, 23}, it is conceivable that differential DNA methylation and consequently different regulation of osmoregulatory genes facilitates local adaptation to salinity. Remarkably, one of the top candidate genes differentially methylated between populations from 20 and 6 PSU was eda (\textit{Ectodysplasin A}), a well-described gene involved in lateral plate formation\textsuperscript{24}. Salinity and calcium are significant drivers of plate morphology\textsuperscript{25} in proposed conjunction with predation\textsuperscript{26}. Our findings suggest that repeated and parallel selection for the low plated eda allele in response to low saline habitats\textsuperscript{27, 28, 29}, including the Baltic Sea\textsuperscript{15, 30}, may involve methylation mechanisms. Taken all together, our results suggest that, along with genetic differentiation, differentially methylated genes likely contribute to local salinity adaptation across stickleback populations (Figure 2, Table 1; Supplementary Material: Figure S2, Table S2A and S2B). To further investigate this hypothesis, we experimentally characterized the proportion of stable (population-specific) and inducible (environment-specific) pop-DMS.

\textbf{Characterizing stable and inducible DNA methylation in a two-generation experiment}

In order to distinguish between stable and inducible DNA methylation we then conducted a two-generation salinity acclimation experiment with laboratory bred sticklebacks from the mid salinity population (Figure 1). We considered pop-DMS to be stable when both the within- and the transgenerational acclimation group were not differentially methylated compared to the control group (\textit{q}-value ≥ 0.0125). These population-specific and environmentally insensitive pop-DMS could be a target for natural selection via the phenotype (sensu \textit{selection-based}'). On the other hand, if a pop-DMS was also differentially methylated between at least one of the acclimation
groups (within- and transgenerational) compared to the control group (q-value < 0.0125; methylation difference ≥ 15%) this site was considered inducible. Such environment-specific pop-DMS can facilitate adaptive transgenerational plasticity in response to environmental changes (sensu detection-based). After two generations of salinity acclimation, we found that the majority of the pop-DMS remained stable, regardless of the direction of salinity change (926 pop-DMS = 63% at decreased salinity; 694 pop-DMS = 60% at increased salinity). A smaller number of pop-DMS (13%) were inducible, as they showed a significant change in CpG methylation upon experimental salinity decrease (198 pop-DMS) or increase (148 pop-DMS). An additional 24% and 27% (346 and 316 pop-DMS respectively) differed significantly between experimental treatment groups, but did not exceed the minimum threshold in differential DNA methylation of 15% employed in this study. Interestingly, the number of inducible pop-DMS (13%) derived from comparisons between natural populations was significantly higher compared to what would be expected from a random selection of CpG sites across the genome (< 1%; 1000 replicates; salinity decrease: $X^2 = 1090.7, P < 0.001$; salinity increase: $X^2 = 967.7, P < 0.001$). This shows that a proportion of the pop-DMS reflects a salinity-mediated plastic response, as it is expected for detection-based sites.

**Associating genes with stable and inducible DNA methylation**

We then assessed both stable and inducible pop-DMS for associations with different putative gene function (Figure 2, Supplementary Figure S2). Genes associated with stable pop-DMS (452 and 329 under salinity decrease and increase, respectively) were enriched for basic biological processes (e.g. DNA repair, chromatid segregation), but also for osmoregulatory functions (e.g. cation and proton channel activity; Figure 2). In line with mathematical models on the role of epigenetic and genetic changes in adaptive evolution, these stable DNA methylation sites were potential targets for natural selection, resulting in differential DNA methylation between locally adapted populations. Inducible pop-DMS were associated with genes (100 and
82 under salinity decrease and increase, respectively) that were primarily enriched for other osmoregulatory functions such as ion channel activity and homeostasis (Figure 2, Supplementary Figure S2). We take this functional association as further evidence that inducible pop-DMS, sensu *detection-based*, are representing a molecular basis of adaptive phenotypic plasticity by allowing individuals to regulate their ion balance relative to the seawater medium instantaneously without requiring any further genetic adaptation at the population level.

**Figure 2:** Gene ontology terms for biological processes and molecular functions.
Gene ontology (GO) terms for biological processes and molecular functions under salinity increase (yellow, 20 vs. 33 PSU) and decrease (blue, 20 vs. 6 PSU) associated with differentially methylated sites between populations (pop-DMS) are presented. The graph is split into GO terms associated with pop-DMS from natural stickleback populations across a salinity cline (wild) and their experimental inducibility (inducible and stable) in a two-generation acclimation experiment. The size of the circles refers to the number of genes of this term present in our groups (in %) and the transparency to the $P$-value (darker circles refer to a lower $P$-value). This subset is filtered for GO terms including the following keywords: "anion", "cation", "channel", "transport", "water", "chloride", "potassium", "homeostasis", "DNA", "chromatid", "chromosome", "spindle", "ectoderm", "endoderm", see Figure S2 (Supplementary Material) for the full figure.
Table 1: Differentially methylated genes across natural populations along a salinity cline.

Genes derived from DNA methylation comparisons between natural populations associated with ≥ 10 pop-DMS (decreased salinity: KIE (20 PSU) vs. NYN (6 PSU); increased salinity: KIE (20 PSU) vs. SYL (33 PSU)). Ensembl gene ID and name as well as the position on the chromosome are listed. The numbers refer to the numbers of DMS in the population comparison (wild). These DMS were classified into ‘inducible’, ‘semi-inducible’ and ‘stable’ sites according to their behavior in a two-generation salinity acclimation experiment with laboratory bred sticklebacks from the mid salinity population (20 PSU) exposed to experimental salinity increase or decrease (33 and 6 PSU respectively). Further, inducible sites were distinguished whether they matched methylation levels of the locally adapted population (‘expected’) or not (‘opposite’). Genes written in bold vary in both population

| Ensembl gene ID | chromosome | start position | end position | gene name       | wild | inducible | ‘expected’ inducible | ‘opposite’ inducible | stable | semi-inducible | Fisher’s exact (P) |
|-----------------|------------|----------------|--------------|----------------|------|-----------|----------------------|----------------------|--------|----------------|-------------------|
| ENSGACG000000008328 | Chr10      | 12860144       | 12863850     | si:dkey-166k12.1 | 24   | 0         | 0                    | 0                    | 9      | 15             | 0.005             |
| ENSGACG000000019416 | Chr7       | 4451892        | 4453656      | HMX1 orthologue | 17   | 0         | 0                    | 0                    | 9      | 8              | 0.033             |
| ENSGACG000000013229 | Chr18      | 15327717       | 15352321     |                | 15   | 0         | 0                    | 0                    | 3      | 12             | 0.011             |
| ENSGACG000000017287 | Chr3       | 13454527       | 13465167     | mmp16b         | 12   | 0         | 0                    | 0                    | 12     | 0              | 0.001             |
| ENSGACG000000017584 | Chr3       | 14690814       | 14944448     | CCNY           | 12   | 12        | 0                    | 0                    | 0      | 0              | 0.001             |
| ENSGACG000000018249 | Chr4       | 12141625       | 12143011     | si:ch211-153b23.5 | 12   | 1         | 1                    | 0                    | 3      | 8              | 0.188             |
| ENSGACG000000008034 | Chr6       | 9368187        | 9380941      |                | 11   | 10        | 0                    | 0                    | 0      | 1              | 0.014             |
| ENSGACG000000009469 | Chr1       | 9166576        | 9173856      | egln2          | 11   | 0         | 0                    | 0                    | 11     | 0              | 0.001             |
| ENSGACG00000004433 | Chr17      | 2127457        | 221376       | igsf21a        | 10   | 10        | 0                    | 0                    | 0      | 0              | 0.003             |
| ENSGACG000000007343 | Chr10      | 1066995        | 10679785     | col9a2         | 10   | 0         | 0                    | 0                    | 6      | 4              | 0.227             |
| ENSGACG000000018407 | Chr4       | 13828336       | 13837518     | Snodb          | 10   | 2         | 2                    | 0                    | 5      | 3              | 0.848             |
|                  | ENSGACG0000000020323 | Chr7       | 17010160     | 1701176        | 23   | 0         | 0                    | 0                    | 22     | 1              | <0.001            |
| ENSGACG000000013229 | Chr18      | 15327717       | 15352321     |                | 15   | 10        | 10                   | 0                    | 22     | 1              | 0.125             |
|                   | ENSGACG000000013359 | Chr11      | 12960883     | 12968110       | 15   | 0         | 0                    | 0                    | 12     | 3              | 0.011             |
| ENSGACG000000019416 | Chr7       | 4451892        | 4453656      | HMX1 orthologue | 15   | 3         | 3                    | 0                    | 5      | 7              | 0.745             |
| ENSGACG000000002948 | Chr8       | 218240         | 221355       | ddx10          | 14   | 0         | 0                    | 0                    | 6      | 8              | 0.077             |
| ENSGACG000000016350 | Chr14      | 3603545        | 3604923      |                | 14   | 1         | 0                    | 0                    | 1      | 7              | 0.277             |
| ENSGACG000000006636 | Chr18      | 4780893        | 4786820      | ZC3H12D        | 13   | 0         | 0                    | 0                    | 3      | 10             | 0.034             |
| ENSGACG000000004667 | Chr12      | 4273498        | 4286193      | tti1           | 12   | 0         | 0                    | 0                    | 12     | 0              | 0.001             |
| ENSGACG000000015566 | Chr2       | 9043062        | 9051779      | casc4          | 10   | 0         | 0                    | 0                    | 10     | 0              | 0.003             |
comparisons. We used a Fisher’s exact test to assess whether pop-DMS associated to the same gene are correlated in their response to experimental salinity change (non-random distribution among the categories stable, inducible, semi-inducible) and reported corresponding $P$-values. For a full table on all genes associated with 1 or more pop-DMS see Table S2A and S2B (Supplementary Material).
Assessing the role of experimentally inducible DNA methylation in nature

We then assessed if multiple pop-DMS associated with the same gene showed a correlated response to experimental salinity acclimation, which would result in a non-random distribution of pop-DMS within genes among the categories ‘stable’, ‘inducible’ and ‘semi-inducible’. We found that in 13 out of 20 genes with ≥ 10 pop-DMS, these pop-DMS responded similarly across the gene upon salinity acclimation (Table 1, Fisher’s exact test, \( P < 0.05 \)). This non-random pattern of change provides additional evidence that we have identified inducible pop-DMS relevant to the detection-based\(^7\) information pathway. Secondly, we tested whether a change at inducible pop-DMS in experimental fish increased the similarity to methylation patterns found in natural populations locally adapted to their respective salinity conditions. Of the 198 (decreased salinity) and 148 (increased salinity) inducible pop-DMS, 130 (66%) and 101 (68%), respectively, changed to become more similar to methylation levels of the locally adapted field populations (hereafter ‘expected’ direction). Conversely, at 68 and 47 inducible pop-DMS experimental fish showed methylation changes in the opposite direction, decreasing the similarity with methylation levels observed in the natural populations from 6 and 33 PSU, respectively (hereafter ‘opposite’ direction). At this point, it is tempting to assume that a DNA methylation change in the expected direction is adaptive, while a change in the opposite direction is maladaptive. However, since correlations exist between genetic variants and DNA methylation\(^{31, 32}\), and SNPs at CpG sites may interfere with methylation function\(^{33, 34}\), conclusive evidence requires additional genomic characterization.

We then hypothesized that opposite inducible pop-DMS are associated with higher genomic (DNA sequence-based) differentiation, while we anticipated the reverse at expected inducible pop-DMS. Accordingly, we re-sequenced whole genomes of the same wild caught individuals we used for RRBS and calculated the degree of genomic differentiation per inducible pop-DMS as mean \( F_{ST} \) value (± 5 kb window) between populations. In line with our hypothesis, the populations from Kiel and Nynäshamn (decreased salinity) were genetically more
differentiated at opposite inducible pop-DMS than at expected sites ($\delta$.mean.$F_{ST}$ = -0.014, $P =$ 0.002; Figure 3A and 3B). A similar trend, yet not significant, was found between the populations from Kiel and Sylt (increased salinity: $\delta$.mean.$F_{ST}$ = -0.005, $P$ = 0.153; Figure 3C and 3D). Here, the lack of significance may be due to increased mortality and hence selection under increased salinity$^{17}$. Thus, at least under decreased salinity, when experimentally induced DNA methylation becomes more similar to the methylation in natural populations, this occurs in a genomic background with low genetic differentiation. On the other hand, when experimentally induced methylation differences to the low salinity population increase (Figure 3A and 3B), this occurs in a genomic background with higher genetic differentiation. This finding underlines the importance of the genomic background when interpreting DNA methylation patterns. We suggest that genomic information at these regions already mirrors past selection leading to DNA-based local adaptation, rendering epigenetic modifications less relevant$^6$. Nevertheless, it remains to be tested what happens to all induced DNA methylation sites with selection over multiple generations.
Figure 3: Differential DNA methylation between populations depends on the degree of genomic differentiation.

Figure 3A and 3C show mean $F_{ST}$ values for pop-DMS (with a ±5 kb window) inducible under experimental salinity decrease (top, blue) and increase (bottom, yellow), that either shifted methylation levels towards the values observed in the field (expected) or the opposite direction (opposite). A randomization test (with 10,000 bootstraps) was performed for the difference between expected and opposite mean $F_{ST}$ value ($\delta_{\text{mean.}F_{ST}} = \text{expected} \text{ mean } F_{ST} - \text{opposite} \text{ mean } F_{ST}$; Figure 3B and 3D).

Under the one tailed hypothesis of increased genetic differentiation at opposite sites and an alpha of 0.05 the $P$-value was calculated as values smaller than the true difference divided by 10,000 bootstraps.
Comparing within- and transgenerational acclimation effects on inducible DNA methylation

We then tested whether or not transgenerational plasticity of DNA methylation is adaptive. Under this hypothesis, salinity acclimation over two consecutive generations compared to only within generation exposure would enhance the similarity at inducible pop-DMS with patterns found among wild populations at corresponding salinities. Hence, we calculated the percentage match (δ.meth.diff, Figure 4) between the within- and transgenerational acclimation groups in relation to the anticipated adaptive methylation level at inducible pop-DMS. We found that transgenerational compared to only within generation salinity manipulation increased the δ.meth.diff (for ‘expected’ inducible methylation: decreased salinity: F₁,256 = 30.42, P < 0.001; increased salinity: F₁,198 = 10.39, P = 0.001; Figure 4A and C). Remarkably, we found an interaction of ‘methylation direction’ (hypermethylation or hypomethylation) and ‘acclimation’ (within- and transgenerational) affecting the δ.meth.diff under decreased salinity (ANOVA, δ.meth.diff ~ methylation direction * acclimation, F₁,256 = 7.69, P = 0.006; Figure 4A). Here, transgenerational acclimation increased the similarity of hypomethylated sites to methylation levels found in natural populations, while hypermethylated sites showed equally similar values within- and across generations (Figure 4A). While for ‘expected’ inducible sites this effect was only present under decreased salinity, at ‘opposite’ inducible sites transgenerational acclimation to decreased and increased salinity enhanced the δ.meth.diff at hypomethylated sites (Figure 4B and D; ANOVA, δ.meth.diff ~ methylation direction * acclimation, decreased salinity: F₁,132 = 19.89, P < 0.001; increased salinity: F₁,90 = 9.85, P = 0.002). Generally, the spontaneous addition of a methyl-group to a cytosine is 2.5 times more likely than the removal making a targeted de-methylation harder to achieve. In the transgenerational acclimation group, the methylation reprogramming including extensive methylation erasure and de novo methylation during gamete formation and zygote development could serve as a mechanistic basis to enhance de-methylation of CpG sites.
Figure 4: Effect of the duration of acclimation (within- vs. transgenerational) on DNA methylation inducibility. The y-axis shows the percentage match between the within- and transgenerational acclimation group in relation to the anticipated adaptive methylation level found in natural populations at inducible pop-DMS. This value was obtained by calculating the difference between the methylation change in the experiment (meth.diff.exp in %; control vs. within-generational or control vs. transgenerational) and the difference in methylation between natural populations (meth.diff.wild in %) as δ.meth.diff = 100 – (meth.diff.wild – meth.diff.exp). Mean values ± 95% confidence interval are shown for within- and transgenerational acclimation to decreased and increased salinity at expected and opposite inducible sites. Colors refer to the direction of DNA methylation change (hypomethylation or hypermethylation). Values closer to 100 indicate a shift in methylation pattern towards adaptive methylation levels found in natural populations and stars indicate the significance level (P ≤ 0.001 ‘***’; P ≤ 0.01 ‘**’) for the comparison between within- and transgenerational acclimation. ‘Main effect’ refers to an
effect of acclimation (within- or transgenerational) and 'interaction effect' to an interaction of acclimation and methylation direction (hypo- or hypermethylation).

Conclusion

Our study provides the first empirical evidence that stable and inducible DNA methylation in nature exist and follow predictions from evolutionary theory according to a selection- and a detection-based epigenetic pathway to promote adaptation to novel environments\(^5,7\) (Figure 5). Consistent with modeled selection dynamics of DNA methylation\(^7\), we identified DMS between populations enriched for critical osmoregulatory functions insensitive to experimental salinity change. Such selection-based (stable) methylation works along the same evolutionary trajectory as adaptive DNA sequence-based evolution\(^5,7\). Given expected epimutation rates of approximately \(10^{-4}\) (estimated for \(A.\ thaliana^{38}\)), the resulting phenotypic variation allows populations to explore the fitness landscape faster than under DNA sequence based genetic variation alone\(^5,35\). On the other hand, other osmoregulatory functions corresponded to the detection-based epigenetic pathway\(^7\) as they were associated with inducible DMS. This inducibility accumulated in the predicted direction across generations and forms a molecular basis of adaptive transgenerational plasticity. The latter has been widely discussed as potential buffer of environmental changes\(^{10,18,39}\) which would allow populations to persist in the face of disturbance by moving phenotypes faster in the direction of optimal fitness than genetic changes alone\(^5,35\). By combining experiments on the inducibility of the methylation level of certain DMS with field observations on populations locally adapted to different salinity regimes\(^{15}\), we gained unprecedented insights into the role of DNA methylation patterns in natural populations. Overall, our study demonstrates that DNA methylation works alongside genetic evolution to facilitate local adaptation and promote adaptive transgenerational plasticity.
Figure 5: Graphical summary of experimental design and main results.

We used the Baltic Sea salinity gradient to study the role of DNA methylation in local salinity adaptation and the response to salinity change in a space-for-time approach. To assess the potential future acclimatization and adaptation processes of the natural stickleback population from 20 PSU (Kiel / green) to the predicted desalination, we compared differences in DNA methylation at CpG sites between wild caught and laboratory bred sticklebacks. Following the experiment timeline (bottom), we compared methylation levels of the experimental control group from 20 PSU, to within- and transgenerational acclimation of 20 PSU sticklebacks to 6 PSU (DNA from left to right). The population locally adapted to 6 PSU serves as the hypothetical future state in which salinities will decrease (blue, DNA on the right). The three main results are written in the circles with schematically and horizontally corresponding DNA methylation changes. (i) 63% of the DMS between the populations remained stable under experimental salinity change. (ii) The direction of experimental methylation change was dependent not only on the treatment but also on the degree of genetic differentiation between the populations (see Figure 3 for...
results). (iii) Transgenerational salinity acclimation shifted DNA methylation patterns closer to the anticipated adaptive state found in the hypothetical future population (see Figure 4 for results). For clarity, only one (6 PSU) of the two foreign salinity regimes tested (6 and 33 PSU) is shown, indicated by the yellow fish on the top left (see Figure 1 for full experimental design).

Material and Methods

Animal Welfare

All catches were performed under legal authorization issued by the German ‘Ministry of Energy Transition, Agriculture, Environment, Nature and Digitalization’ in Schleswig-Holstein (MELUR – V242-7224.121-19), by the Danish ‘Ministry of Food, Agriculture and Fisheries of Denmark’ (Case no: 14-7410-000227), by the Estonian ‘Ministry of the Environment’ (Keskkonnaministeerium - eripüügiluba nr 28/2014) and by the Swedish Sea and Water Authority (Havs och Vattenmyndigheten). Ethical permission for the experiments required by German law was given by the MELUR: V312-7224.121-19).

Survey and experimental design

For the field survey, we collected juvenile three-spined sticklebacks (*Gasterosteus aculeatus*; 31.68 ± 14.25 mm) from three different salinity regimes inside and outside the Baltic Sea (Sylt (SYL), Germany (55°00’58.3”N, 8°26’22.0”E), 33 PSU, *N* = 16; Kiel (KIE), Germany (54°26’11.8”N 10°10’20.2”E), 20 PSU, *N* = 16; Nynäshamn (NYN), Sweden (58°52’44.7”N 17°56’06.2”E), 6PSU, *N* = 16) in September 2014. Once collected, fish were immediately euthanized using tricaine methane sulfonate solution (MS222), photographed, measured (length and total weight) and stored in RNA-later (24h at 7°C, afterwards at -20°C). A cut along the ventral side ensured that the RNA-later solution would diffuse into all tissues. Conserved specimen were later dissected in the lab and gill tissue was separated. For the acclimation experiment, we collected live adult fish from Kiel (20 PSU), which were crossed in our facilities at
GEOMAR to obtain ten F1 laboratory bred families, herein referred to as ‘parental generation’. At
nine months post-hatch we split each family into three salinity treatment groups of 10 fish each:
one at 33 PSU, one at 6 PSU, and one control group at 20 PSU. The salinity transition was
performed within 10 days by 3 PSU steps every second day. Over the entire time each group
was fed ad libitum and kept in a 20-L aquarium connected to one of three filter tanks per salinity
treatment. After 5 months under treatment conditions, six pure crosses per salinity treatment
group were performed in vitro, herein referred to as ‘offspring generation’ (F2). Upon fertilization,
clutches were split and separated into different treatments (Figure 1). At three months post-
hatch, laboratory bred F2 sticklebacks were euthanized using MS222, photographed, dissected
and their gill tissue was stored in RNA-later. The age at sampling matched the estimated age of
the wild caught juveniles. Additionally, to the 48 wild caught individuals from Kiel, Nynäshamn
and Sylt that were used in above field survey, we sequenced whole genomes from gill tissue of
an additional three populations of sticklebacks, namely from Falsterbo, Sweden (55°24'46.6"N
12°55'52.3"E; 10 PSU; N = 16), Letipea (59°33'07.6"N 26°36'29.7"E; 4 PSU; N = 16) and Barsta
(62°51'47.1"N 18°23'51.0"E; 5 PSU; N = 16).

**DNA extraction**

For the field survey, DNA extraction of gill tissue (N = 16 individuals per population) was
performed using the DNeasy Blood & Tissue Kit (Qiagen). Further purification of the extracted
DNA was done with NucleoSpin® gDNA Clean-up (Macherey-Nagel). For laboratory bred F2
offspring of the two-generation acclimation experiment, dual extraction of whole RNA and DNA
was performed from gill tissue (N = 11-12 individuals per treatment group, Figure 1) stored in
RNAlater using the AllPrep DNA/RNA mini kit (Qiagen). Purity and quality of the extracted DNA
was estimated using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and a
standard agarose gel (1% Agarose/TAE). DNA concentration was assessed using the Qubit®
2.0 Fluorometer (Thermo Fisher Scientific). To obtain a balanced sex ratio, we determined the
gender of the individuals using a sex-specific genetic polymorphism in isocitrate dehydrogenase (IDH) with a modified protocol from Peichel et al. (2004)\textsuperscript{41}. For the PCR (settings: once 94°C for 3 minutes; 30 cycles of 94°C for 30 sec, 54°C for 20 sec, 72°C for 30 sec; once 72°C for 5 minutes), 1 µL forward and reverse primer (5µM) was used with 4.9 µL water, 1 µL 10x buffer, 1 µL dNTPs (0.5 µM), and 0.1 µL DreamTaq (5 U/µL). The resulting PCR products were visualized with a capillary electrophoresis on the 3100 ABI sequencer and a LIZ500 size standard. While males show a heterogametic signal with two bands (at approximately 300 bp and 270 bp), females lack the band at 270 bp.

**Library preparation and sequencing (Whole genome sequencing, WGS)**

For whole genome sequencing, the ‘TruSeq Nano DNA’ (Illumina) library preparation kit was used according to the manufacturer’s protocol by the Sequencing Facility of the IKMB, University of Kiel. Ultrasonication was conducted with a ‘Covaris E220’ (Covaris) to shear the input DNA (100 ng per sample and 350 bp insert size). Before the enrichment with a PCR step (8 cycles), fragmented and bead purified DNA was ligated with adenylate at the blunt 3’ ends (End repair and A-tailing) and with indexing adapters. Fragments were cleaned with MagSi-NGS Prep Plus Beads (Steinbrenner). Paired-end sequencing of the quality-controlled and multiplexed libraries was performed on the Illumina Hiseq 4000 platform (2 x 150 bp reads).

**Quality assessment, data filtering and mapping (WGS)**

The command line tools of *Picard v.2.7.1* (Broad Institute 2016) were used to (i) reformat the Fastq to uBAM file format and to add further values (read group etc.) to the SAM header using *FastqToSam*, to (ii) mark the location of adapter sequences using *MarkIlluminaAdapters*, and to (iii) reconvert the sequences to Fastq format with *SamToFastq*. The stickleback genome (here Broad/gasAcu1) was indexed with *bwa index* and used as a reference for the mapping with *bwa*
mem\textsuperscript{42} v.07.12-r1044. To retain the meta-information from the uBAMs we used MergeBamAlignment. Picard was also used to identify duplicates with MarkDuplicates. Basic statistics were generated with CollectWgsMetrics, CollectInsertSizeMetrics and AlignmentSummaryMetrics and summarized with MultiQC\textsuperscript{43}. A total number of 4,463,070,154 high-quality reads (mapping quality > Q20) was mapped resulting in a mean depth of 13.84x (sd. 2.02x) and mean insert size 383.07 bp (sd. 9.40 bp, Supplementary Table S3). GATK v 3.7 HaplotypeCaller\textsuperscript{44} was run to determine the likelihoods of the haplotypes per sample, i.e. to call SNPs and indels, which were than processed with GenotypeGVCFs for a joint genotyping. SNPs were selected using hard filters for quality and extracted from the raw genotypes with a combination of the SelectVariants, VariantsToTable and VariantFiltration commands. VCFtools\textsuperscript{45} was used in a next step, removing SNPs with a minimum quality score ($minQ$) below 20 and a minor allele frequency (maf) greater than or equal 0.0049.

**Library preparation and sequencing (reduced representation bisulfite sequencing, RRBS)**

The library preparation for methylation analyses followed the Smallwood and Kelsey reduced representation bisulfite sequencing (RRBS) protocol\textsuperscript{46}. A total of 100-250 ng purified DNA was digested with the methylation-insensitive MspI restriction enzyme, which cuts at the “CCGG” motif and thereby enriches for CpG regions. DNA end-repair and A-tailing was conducted and un-tailed CEGX spike-in controls (Cambridge Epigenetix) were added. These are DNA oligos of known sequence and with known cytosine modification, which can be used for downstream assessment of bisulfite conversion efficiency. After adapter ligation, bisulfite conversion was conducted using the EZ-96 DNA Methylation-Gold Kit (Zymo Research) according to the manufacturer’s protocol. PCR amplification with 19 cycles were performed. Quality control of purified PCR products was performed on a 2200 TapeStation System (Agilent) and high-quality libraries were pooled and diversified with 15% PhiX. Single-end sequencing with 100 bp read length was conducted on a HiSeq 2500 sequencer (Illumina).
Quality assessment, data filtering and mapping (RRBS)

In total 106 individuals (48 wild caught and 58 experimental fish) of balanced sex ratio were DNA sequenced at an average of 19.8 ± 3.5 million reads for experimental fish and 11.4 ± 2.1 million reads for wild-caught fish (Supplementary Table S4). De-multiplexed fastq files were quality checked using FastQC v0.11.5 and Multiqc v1.3. Adapters were removed with cutadapt v1.9.1 using multiple adapter sequences (NNAGATCGGAAGAGCACAC, AGATCGGAAGAGCACAC, ATCGGAAGAGCACAC) with a minimum overlap of one base pair between adapter and read. This was necessary to remove primer dimers and avoid false methylation calls systematically caused by the RRBS end-repair step during library preparation, if the end repair step adds artificial cytosins. Simultaneously, cutadapt was used to trim low quality bases (-q 20) from the 3'-end and remove trimmed reads shorter than 10 bases. An air bubble during sequencing caused the bases 66-72 of ten tiles of one lane (affecting 12 individuals) to have low quality values, which were removed in a custom awk script. Two poor quality individuals (a Sylt and a Nynäshamn female) did not meet our strict quality requirements (e.g.: ≥ 5 million reads, mapping efficiency > 52%) and showed biases in the proportion of bases per position compared to other individuals (plot in fastqc "per base sequence content"). Therefore, we excluded these two libraries from downstream analysis resulting in 15 instead of 16 individuals from Sylt and Nynäshamn (Figure 1). Bisulfite conversion efficiency was assessed from the spike-in controls (Cambridge Epigenetix), using the cegxQC software. Overall conversion levels were 2.4 ± 1.8% conversion of methylated cytosines and 99.6 ± 0.5% conversion of un-methylated cytosines, which is in line with expected conversion rates (Supplementary Table S4). We used Bismark v0.17.0 to index the UCSC stickleback reference genome (Broad/gasAcu1) and to generate the bisulfite alignments with Bowtie2 v2.3.3 at default settings. Bismark was also used to extract the methylation calls. Average mapping efficiency was 63.7 ± 2.4% (Supplementary Table S4).
Identification of differentially methylated sites

The methylation calls were analyzed in R v3.4.1\textsuperscript{51} using the package methylKit v1.3.8\textsuperscript{52}. CpG loci were filtered for a minimum coverage of 10 reads per site. To account for potential PCR bias, we additionally excluded all sites in the 99.9\textsuperscript{th} percentile of coverage. To improve the methylation estimates, we corrected for SNPs, which could have led to a wrong methylation call. The excluded positions were derived with custom written perl scripts from C-to-T and G-to-A-SNPs with genotype quality of 20 and a minimum allele frequency of 0.005 (see above) from the 96 wild caught individuals with a combination of custom written Perl and R-scripts using packages from methylkit\textsuperscript{52} and GenomicRanges\textsuperscript{53} (Supplementary File Summary\_scripts.txt).

After normalizing coverage values between samples, using normalizeCoverage implemented in methylKit, we excluded all sites that were present in fewer than 9 individuals per treatment group from downstream analysis. As previously shown, sex specific methylation affects < 0.1\% of CpG sites on autosomal chromosomes, but > 5\% of CpGs on the sex chromosome\textsuperscript{19}. Therefore, to exclude a potential sex bias, we removed all CpG sites located on the sex chromosomes (chromosome 19), resulting in a high-quality dataset with 525,985 CpG sites. Finally, by checking the first six principal components of the resulting PCA and running an ANOVA on the filtered dataset, we confirmed the absence of an effect of sex on global methylation pattern ($F_{124,1} = 2.611, P = 0.109$). However, the PCA revealed a bias in methylation pattern by families over all experimental groups. Therefore, to identify differentially methylated CpG sites (DMS) between treatment groups, we performed pairwise comparisons (Supplementary Table S5) fitting a logistic regression model per CpG site with calculateDiffMeth in methylKit using family as covariate for the experimental groups. A Chi-square test was applied to assess significance levels of DMS and $P$-values were corrected to $q$-values for multiple testing using the SLIM (sliding linear model) method\textsuperscript{54}. Additionally, we accounted for multiple use of groups in pairwise comparisons and adjusted the alpha for the $q$-value according to Bonferroni correction to 0.0125.
Ultimately, CpG sites were considered to be differentially methylated with a $q$-value $< 0.0125$ and a minimum weighted mean methylation difference of 15%. To ensure that the DMS obtained are not laboratory artefacts, we used `calculateDiffMeth` implemented in `methylKit` compared the wild population from Kiel to the experimental control group (Kiel population from 20 PSU at 20 PSU). The resulting 11,828 DMS were excluded from the DMS obtained by the pairwise comparisons mentioned above (Supplementary Table S5). DMS were plotted across the genome for the comparison between Kiel vs Nynäshamn (20 vs. 6 PSU, blue fish) and Kiel vs Sylt (20 vs. 33 PSU, yellow fish) using `ggplot2` and `hypoimg` (Supplementary Figure S3).

**Assessment of inducibility and gene association of DMS**

By comparing wild caught individuals from the mid salinity population (KIE, 20 PSU) to the populations sampled at low (6 PSU, NYN) and high (33 PSU, SYL) salinity in the field, we obtained 1,470 (KIE-NYN) and 1,158 (KIE-SYL) pairwise pop-DMS, which are hypothetically involved in local adaptation. We first tested whether these pop-DMS distinguishing natural populations are inducible or stable at the respective salinity in the experiment. A pop-DMS was considered stable when the within- and the transgenerational acclimation group did not significantly differ in methylation to the control group ($q$-value $\geq 0.0125$). On the other hand, pop-DMS were considered inducible when at least one of the acclimation groups was differentially methylated compared to the control group ($q$-value $< 0.0125$; methylation difference $\geq 15\%$). Pop-DMS with a significant $q$-value not exceeding the threshold of differential DNA methylation (15%) will be referred to as `semi-inducible` hereafter. We used a randomization test to ensure that the number of inducible sites obtained did not occur by chance. To this end, we randomly sampled 1,470 (KIE-NYN) and 1,158 (KIE-SYL) pop-DMS from the complete dataset (1,000 replicates). A Chi-square test was used to assess whether our observed number of inducible, stable and semi-inducible sites differs from a random set of sites (averaged over replicates). Finally, we tested whether the weighted mean methylation difference (meth.diff, in percentage)
between wild populations matches the inducible methylation difference by subtracting the 'meth.diff' in the experiment (exp) from the 'meth.diff' between wild caught populations (wild):

\[ \delta.meth.diff = 100 - (meth.diff.wild - meth.diff.exp) \]

As we subtracted this difference from 100, values closer to 100 indicated higher similarity of experimentally inducible methylation with the postulated adaptive DNA methylation pattern in natural populations. By comparing the '\( \delta.meth.diff \)' for within- and transgenerational acclimation using an ANOVA, we can assess whether there is a difference in inducibility of methylation to match patterns found in wild caught populations. All analyses were run separately for decreased (6 PSU; KIE-NYN) and increased (33 PSU; KIE-SYL) salinity.

In order to detect potential functional associations of the observed changes in DNA methylation state, we classified the genomic region of a pop-DMS based on their nearest transcription start site (TSS) using `annotateWithGeneParts` and `getAssociationWithTSS` implemented in `genomation v1.4.2`\(^7\). We distinguished between promoter (1500 bp upstream and 500 bp downstream of TSS), exon, intron and intergenic regions. To be associated to a gene, the pop-DMS had to be either inside the gene or, if intergenic, not further than 10 kb away from the TSS. We excluded three pop-DMS that were on a different reference scaffold then the gene they were associated to on the chrUn linkage group (that merges scaffolds into one large artificial chromosome). Using the genes with associated pop-DMS, we applied a conditional hypergeometric GO term enrichment analysis (\(P\)-value ≤ 0.05) with the ensembl stickleback annotation dataset ‘`gaculeatus_gene_ensembl`’ and all genes that were associated to any sequenced CpG site as universe. We identified overrepresented biological processes, molecular functions and cellular components using the package `GOstats v2.46`\(^8\) in `R` v3.4.1\(^1\)\(^5\). Figures were produced using `ggplot2`\(^2\)^.\(^5\)

**Estimation of DNA sequence based genetic differentiation at differentially methylated sites**
In order to evaluate the genetic differentiation up- and downstream (in sum 10 kb) of the pop-
DMS position, we calculated the mean $F_{ST}$ values ($\leq 40\%$ missing data and depth $\geq 5$) from
whole genome sequencing data of the exact same individuals with vcftools v.0.1.15\textsuperscript{59}. We
hypothesized that inducible CpG positions matching the methylation difference \textit{expected} from
the profile of the wild populations are genetically more similar between the populations than sites
that changed in the \textit{opposite} direction. To test this one-tailed hypothesis we applied a
randomization test (with 10,000 bootstraps) on the mean $F_{ST}$ difference between the two groups
(expected and opposite):

$$\delta\.mean.F_{ST} = \text{‘expected’ mean } F_{ST} - \text{‘opposite’ mean } F_{ST}$$

We plotted the 10,000 delta mean $F_{ST}$ values and calculated a $P$-value by dividing the proportion
of values smaller than the true difference by the number of bootstraps. Figures were produced
using \textit{ggplot}\textsuperscript{25}.

\textbf{Author contributions}

CE and TBHR conceived and designed the study with contributions for the bisulfite sequencing
strategy from BSM. BSM and MJH planned and carried out the fieldwork at the German
locations, BSM supervised the sampling in Estonia and Sweden, which was carried out by a
great team of the BONUS-BAMBI project. MJH, with the help from CE and TBHR, planned and
supervised the breeding and acclimatization experiment. MJH and BSM conducted wet
laboratory work (DNA extractions and quality assessment). MPH and RH and conducted the
library preparation and sequencing. MJH and BSM analyzed the data and drafted the
manuscript together with equal contributions. All co-authors discussed and interpreted the
results and contributed to the final version of the manuscript.

\textbf{Data Availability}

Fastq raw reads of genomes and methylation sequencing will be deposited in GenBank.
Code Availability

Custom code is available as supplementary information (Summary_scripts.txt).

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Competing interests

The authors declare no competing interests.

Supplementary Information
Methodological details, scripts, supplementary results, figures and tables are provided.

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