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Persistent and plastic effects of temperature on DNA methylation across the genome of threespine stickleback (*Gasterosteus aculeatus*)

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Epigenetic mechanisms such as changes in DNA methylation have the potential to affect the resilience of species to climate change, but little is known about the response of the methylome to changes in environmental temperature in animals. Using reduced representation bisulfite sequencing, we assessed the effects of development temperature and adult acclimation temperature on DNA methylation levels in threespine stickleback (*Gasterosteus aculeatus*). Across all treatments, we identified 2130 differentially methylated cytosines distributed across the genome. Both increases and decreases in temperature during development and with thermal acclimation in adults increased global DNA methylation levels. Approximately 25% of the differentially methylated regions (DMRs) responded to both developmental temperature and adult thermal acclimation, and 50 DMRs were common to all treatments, demonstrating a core response of the epigenome to thermal change at multiple time scales. We also identified differentially methylated loci that were specific to a particular developmental or adult thermal response, which could facilitate the accumulation of epigenetic variation between natural populations that experience different thermal regimes. These data demonstrate that thermal history can have long-lasting effects on the epigenome, highlighting the role of epigenetic modifications in the response to temperature change across multiple time scales.

1. Introduction

Environmental temperature is a critical factor that determines the distribution and abundance of organisms [1–4], and the rapidly changing thermal environment due to global climate change is projected to have a particularly pronounced effect on ectothermic species such as fishes [1,4]. Phenotypic plasticity plays an important role in the responses of organisms to rapid changes in the environment, and could be critical in determining the ability of species to cope with climate change [1,5,6]. Plastic responses can persist across multiple time scales, from short-term and largely reversible acclimation responses, to longer-term developmental plasticity, to transgenerational responses [7], but plasticity at these different time scales often impacts similar physiological processes. For example, in fishes, temperature changes during development can have persistent effects on sex determination, metabolism, thermal performance, muscle phenotypes and gene expression [8–11]. Similarly, exposure of adults to short-term temperature change can induce temporary plastic responses in many of these traits [3,8,12,13]. Physiological plasticity is often modulated at the level of gene expression, thus investigating the regulatory mechanisms controlling gene expression is fundamental to understanding how species cope with climate change [1].

Epigenetic modifications can influence the plasticity of organisms by regulating gene expression without modifying the DNA sequence [14]. DNA methylation, the addition of a methyl group to a cytosine nucleotide, is one of the best-characterized

epigenetic processes. The addition or removal of DNA methylation can be dynamic, occurring rapidly in response to environmental cues [15–17]. DNA methylation patterns can also persist through cell division and be passed on through multiple generations, potentially influencing evolutionary processes and fitness [18,19]. The intra- and intergenerational effects of DNA methylation and the role of DNA methylation in regulating plasticity is an intriguing system in which to examine how epigenetic processes modulate plastic and persistent phenotypic variation in response to changing environments. How this epigenetic system of 'soft inheritance' [20,21] functions as a response to changes in temperature has been highlighted by studies in plants [22–29], yet little is known about these processes in ectothermic vertebrates [30].

To understand how DNA methylation is modified in response to environmental temperature in vertebrates, we used threespine stickleback (*Gasterosteus aculeatus*), an important vertebrate model for studies of ecological adaptation [31,32], in which phenotypic effects of both developmental temperature and adult acclimation temperature have been clearly demonstrated [13,33–38]. By comparing DNA methylation patterns between threespine stickleback with different thermal histories during development to methylation patterns in adult threespine stickleback acclimated to differing temperatures, we demonstrate that epigenetic mechanisms are a component of both persistent and plastic responses to environmental change.

2. Material and methods

(a) Experimental design

Adult threespine stickleback (*G. aculeatus*) were collected from a fully plated 'marine' population in Oyster Lagoon (British Columbia, Canada; GPS: 49.6121, −124.0314) in June 2014. Stickleback were separated into six 110 l glass tanks (20 stickleback per tank) and acclimated for three weeks to 20 ppt salt water (dechlorinated Vancouver municipal tap water supplemented with Instant Ocean Sea Salt), 18°C and 14 : 10 h light : dark photoperiod, which mimics the natural environmental conditions at the collection location at the time of collection. Stickleback were fed daily to satiation with Hakari Bio-Pure frozen Mysis shrimp.

After the three weeks of laboratory acclimation, eggs were collected from six gravid females and testes were dissected from six males and individually macerated in a 1.75 ml microcentrifuge tube containing 150 µl Ginzberg's fish Ringer's solution. Eggs collected from a single female were arranged as a monolayer in Petri dishes containing 5 ml of 20 ppt salt water. 50 µl of the sperm solution from a single male was applied directly on the egg mass and left for 30 min at the acclimation conditions to allow fertilization to occur. This process was repeated six times using six different females and six different males to generate six independent families. Following fertilization, an additional 10 ml of 20 ppt saltwater was added to each Petri dish. Each clutch was then split across three separate Petri dishes. A single Petri dish from each family was transitioned to a developmental temperature of 12°C, 18°C or 24°C (such that all six families were represented in each developmental temperature treatment). Petri dishes were partially covered to prevent water loss via evaporation and to allow for surface gas exchange. Eggs were monitored twice daily, during which time any unfertilized eggs were removed and 10 ml of water was changed to prevent mould growth. Hatching time differed by several days between stickleback reared at different development temperatures (electronic supplementary material, figure S1). After hatching, embryos were moved to 110 l glass aquaria at the original acclimation conditions of 18°C with a 14 : 10 h light : dark photoperiod. Each family was held in a separate mesh breeding box, with two breeding boxes (i.e. two families) per glass aquaria. Aquaria were equipped with hanging box filters (Aquaclear) and sponge filters for filtration and aeration. Larvae were fed live brine shrimp nauplii twice daily ad libitum until they were large enough to feed on frozen Mysis shrimp. Once juvenile stickleback reached a size of approximately 1 cm they were released from the breeding boxes into the aquaria. For the experiment on the effects of developmental temperature on DNA methylation, families were kept separate (two families per split aquarium) until sampling, which occurred at 10 months of age. A total of six stickleback from each development temperature treatment were euthanized and muscle tissue samples (from behind the dorsal fin to the base of the tail) were taken from each stickleback (four females and two males from each treatment except for the 12°C development group which had five females and one male), with sampling distributed across families (electronic supplementary material, table S1). This experimental design minimizes the potential for effects of genetic variation and sex on DNA methylation patterns [39,40].

For the experiment on the effects of adult thermal acclimation on DNA methylation patterns, after nine months of development, a random sample of stickleback from the six families that had developed at 18°C were mixed together and then split between three different acclimation temperatures (5°C, 18°C or 25°C). Following four weeks of thermal acclimation, a total of six sticklebacks from each acclimation temperature treatment were euthanized and muscle tissue was sampled as described above (from four females and two males from each treatment). Muscle tissue was immediately snap frozen in liquid nitrogen and stored at −80°C until further use.

The developmental and acclimation temperature protocols described above created five different groups (four treatments and one control) as follows: stickleback developed at 12°C and acclimated to 18°C, stickleback developed at 24°C and acclimated to 18°C, stickleback developed at 18°C and acclimated to 5°C, stickleback developed at 18°C and acclimated to 25°C, and stickleback developed at 18°C and acclimated to 18°C, the last being treated as the control group (figure 1).

(b) Reduced representation bisulfite sequencing

We chose to examine methylation patterns in muscle tissue because previous work [8,13] has indicated that both developmental temperature and acclimation temperature alter muscle gene expression in fish. Genomic DNA was isolated from muscle tissue using a *Quick*-DNA Miniprep kit (Zymo Research). Bisulfite-treated reduced representation genomic DNA libraries were prepared by the UBC Nucleic Acid and Protein Service core facility and sequenced at the UBC Biodiversity Centre sequencing facility. Libraries were created using a Bio-O NEXTflex Bisulfite-Seq Kit on MspI digested gDNA. Samples were barcoded using NEXTflex Bisulfite-seq Barcodes. Purified, adapter-ligated DNA was then bisulfite treated using an EZ DNA Methylation Gold Kit (Zymo Research). Samples were split between two lanes (three samples per treatment per lane for 15 samples total per lane) of an Illumina HiSeq 2000. Average sequencing library size was 19 900 578 ± 3 880 665 (mean ± s.d.) million reads and covered an average of 12 901 548 CpG sites per sample.

(c) Quantification and statistical analysis

Sequences from the bisulfite-treated samples were aligned to the stickleback genome (Ensembl release 87) and annotated using CLC GENOMICS WORKBENCH v. 9.5 with the bisulfite sequencing plugin v. 1.1.1. Average mapping efficiency was 88.3%. Total coverage and methylated cytosine coverage data were exported and analysed for differential methylation in R v. 3.3.1 using *methylKit* package v. 1.1.3 [41]. Prior to global DNA methylation analysis, CpG loci were filtered so that only sites with at least eight reads were retained in each sample. Sites that were in the

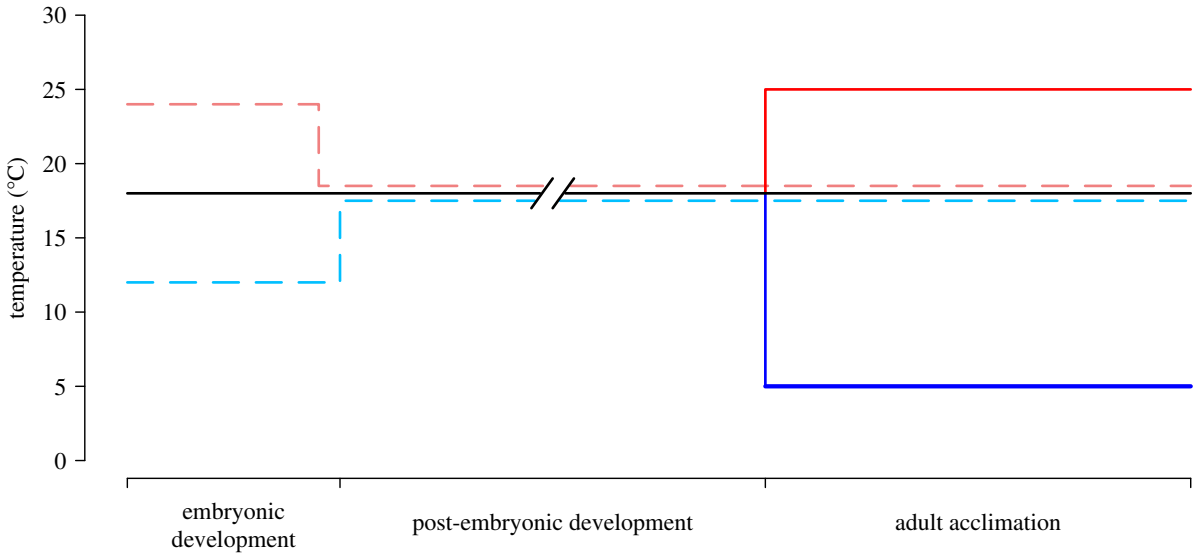

Figure 1. Experimental design. Solid lines represent stickleback that were developed and acclimated to 18°C (black) or that developed at 18°C and were acclimated to 25°C (red) or 5°C (dark blue). Dashed lines represent the developmental temperature treatments of 24°C (pink), or 12°C (light blue). Note that altered development temperature changes time to hatch by several days. Post-hatch, stickleback were transferred to 18°C where they developed to adults for the remainder of the experiment (10 months).

99.9th percentile of coverage were also removed from the analysis to account for potential PCR bias. After filtering, an average of 137 954 CpG sites (1% of all CpG sites in the genome) were retained per library with a mean coverage depth of 15 reads.

To test for differential methylation at specific loci an additional filter was applied such that only CpG loci with at least eight reads in at least four of the six samples within a treatment were kept. Threespine stickleback that were kept at 18°C for the duration of the experiment were used as a reference group for a pairwise comparison between each of the developmental and acclimation temperature treatments.

Mean and median DNA methylation values for each individual were compared using a non-parametric Kruskal–Wallis test. A Kolmogorov–Smirnov test was performed to test for differences in the shape of the cumulative methylation distributions between treatments. All statistical analyses were performed in R v. 3.3.1.

To identify differentially methylated regions (DMRs), the genome was divided into 100 bp regions using the tileMethylCounts() function in *methylKit* v. 1.1.3 with a window size of 100 and a step size of 100. Logistic regression was implemented using the calculateDiffMeth() function to identify differentially methylated loci and DMRs. *p*-values were false discovery rate corrected to *q*-values using the sliding linear model (SLIM) method [42].

To determine the common and unique DMRs among treatment groups, the data were first filtered to include only those regions that were present in the reduced representation sequencing data with at least eight reads in four of the six individuals in every group. Unique and overlapping DMRs were then visualized using the R-package *VennDiagram*. Annotations for DMRs were obtained using the annotatePeakInBatch() function in the R package *ChIPpeakAnno* v. 3.6.5 from BIOCONDUCTOR [43]. GO annotations were obtained from a previous study [44]. Enrichment analysis was performed using *goseq* v. 1.24 [45] and *p*-values were false discovery corrected for multiple comparisons [46].

3. Results and discussion

(a) Temperature change induces genomic hypermethylation

We identified a total of 2130 CpG loci in the stickleback genome that were differentially methylated in response to temperature in at least one treatment. Pairwise comparison of DNA methylation levels at individual CpG loci in stickleback from developmental and adult acclimation treatments to the control (18°C) group identified 554 differentially methylated cytosines (DMCs) in stickleback that developed at 12°C, 480 DMCs in stickleback that developed at 24°C, 1150 DMCs in stickleback that were acclimated to 5°C and 778 DMCs in stickleback acclimated to 25°C. There was no apparent clustering of DMCs on a specific chromosome or chromosomal region (figure 2*a–d*), and DMCs were distributed across promoters, introns, exons and intergenic regions proportionally to the distribution of these features within the genome (electronic supplementary material, figure S2).

Approximately 2–8% of the sequenced CpG sites on each chromosome exhibited differential methylation in response to altered temperature (figure 2*e,f*). More DMCs were hypermethylated than were hypomethylated in all treatments, suggesting that an increase in genomic DNA methylation levels is a general response to changes in environmental temperature (figure 2*e,f*). An increase in genomic DNA methylation levels was expected in stickleback exposed to cooler temperatures based on previous reports of latitudinal variation in genomic DNA methylation levels in fishes, in which polar fishes exhibit higher global DNA methylation levels than do equatorial fish [47]. However, the observed increase in global DNA methylation levels in stickleback exposed to higher temperatures is in contrast to the interspecific correlations between DNA methylation levels and ambient temperature [47].

To further investigate the effects of temperature on genomic DNA methylation levels we examined the mean DNA methylation level for all individuals. Mean genomic methylation values ranged from 72.03% to 73.96%, and median methylation levels ranged from 80.3% to 84.12% (electronic supplementary material, table S2), but the treatment groups were not detected as significantly different from the controls. We also compared the cumulative distribution curves of genomic DNA methylation levels for each of the treatment groups to those of the 18°C control group using the Kolmogorov–Smirnov (K-S) test. Results from the K-S test indicate that the distribution of genomic DNA methylation levels for all treatments was

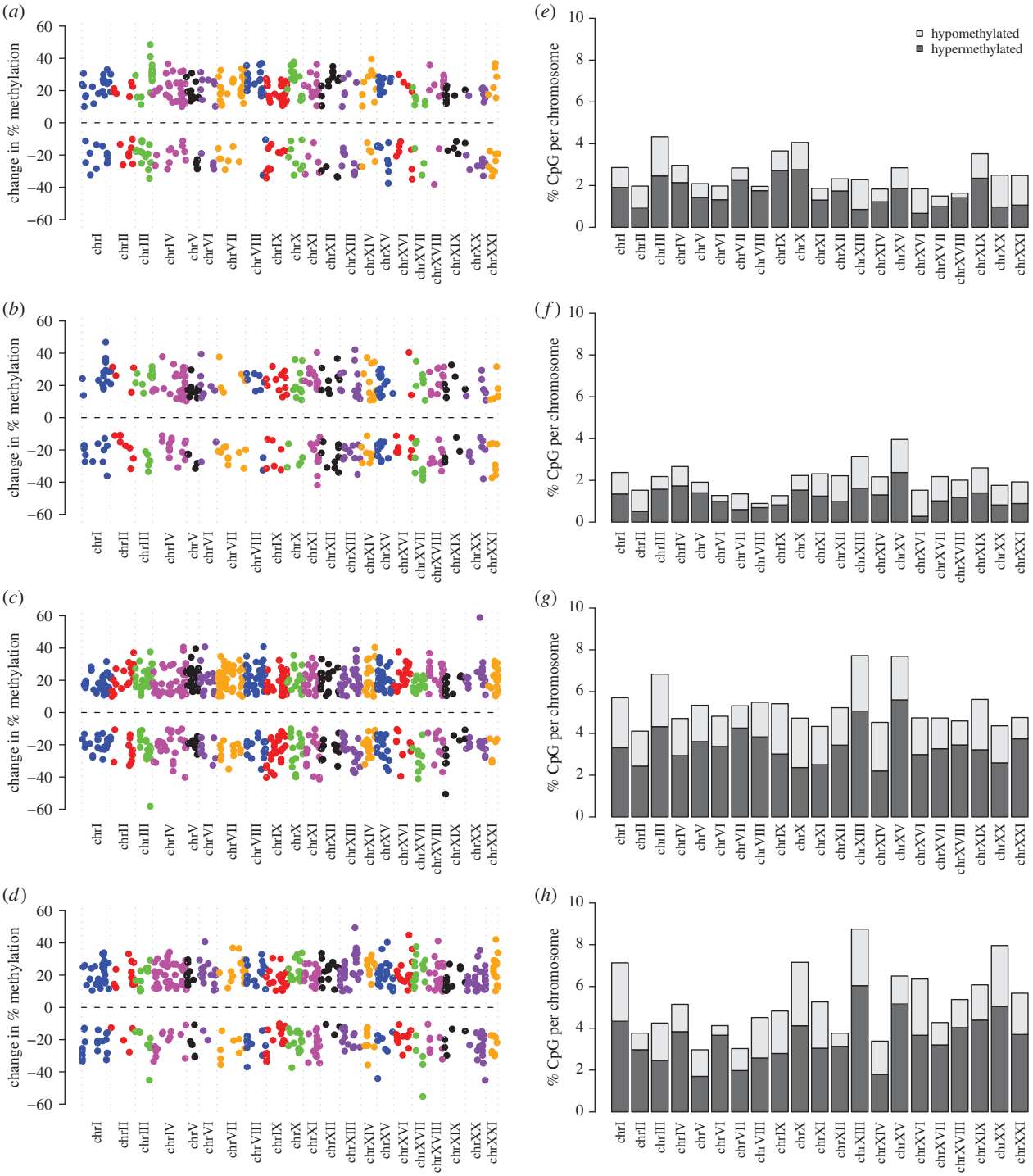

Figure 2. (*a–d*) Manhattan plots of the chromosomal positions of methylated CpG loci that differed significantly between stickleback that were developed at and acclimated to 18°C, and stickleback developed at 12°C (*a*) or at 24°C (*b*) then reared at 18°C, or that were developed at 18°C then acclimated to 5°C (*c*) or 25°C (*d*). Each point represents a single differentially methylated cytosine (DMC). The *y*-axis presents the difference in percentage methylation for that DMC relative to the stickleback developed and acclimated at 18°C. Only DMCs with more than 10% change in methylation are shown. Points above and below the horizontal dashed line are hypermethylated and hypomethylated loci, respectively. Points indicate chromosomal locations. Vertical dashed lines and colours indicate different chromosomal regions. (*e–h*) Chromosomal frequency distribution of hypermethylated (dark grey) and hypomethylated (light grey) DMCs in stickleback that developed at 12°C (*e*) or developed at 24°C (*f*), or that were acclimated to 5°C (*g*) or acclimated to 25°C (*h*). The vertical axis represents the per cent of CpGs that were sequenced that were differentially methylated.

significantly different compared with stickleback from control conditions ($p < 2.2 \times 10^{-16}$; figure 3*a*,*b*) and that the distribution was the most different in stickleback acclimated to 25°C, which had the largest K-S test D statistic (0.05126; electronic supplementary material, table S3). Visual inspection of the violin plots and cumulative distribution curves for DNA methylation (figure 3*a*,*b*) suggest that, in general, these differences are driven by shifts towards increased proportions of highly methylated CpG loci relative to control conditions, which is consistent with the biases towards hypermethylation that we observed among DMCs for all treatments.

Taken together these data clearly illustrate that both developmental temperature and acclimation temperature alter the methylome of threespine stickleback. Changes in DNA methylation have the potential to causes changes in transcriptional regulation, and thus in cellular and organismal phenotypes,

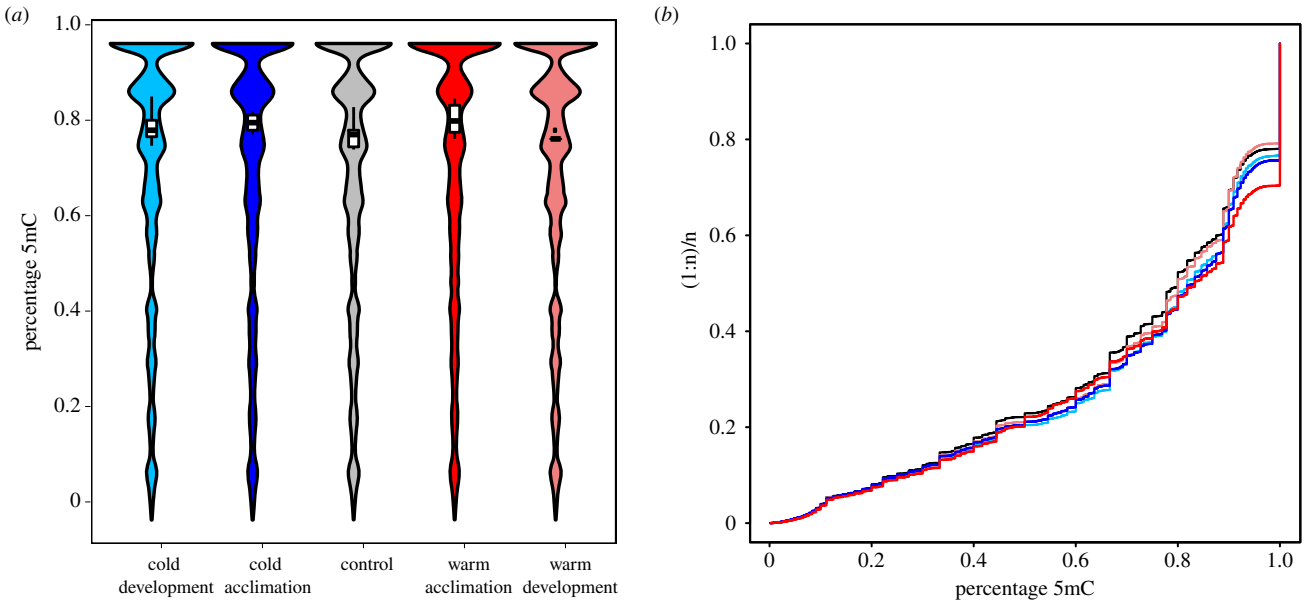

Figure 3. (*a*) Violin plot of genomic DNA methylation levels for each treatment condition and control condition. Colours depict different treatments: stickleback developed and acclimated to 18°C (grey), stickleback developed at 18°C and acclimated to 25°C (red) or acclimated to 5°C (dark blue), and stickleback with a developmental temperature of 24°C (pink) or 12°C (light blue). Width indicates the pooled distribution density of percentage methylation of CpG loci in a given treatment. Embedded box plots summarize variation in the median methylation level across the six samples in each treatment. The line indicates the median of these medians, the box defines the interquartile range (IQR), and the whiskers represent the maximum and minimum values, excluding values greater than 1.5× IQR (which are shown as individual points). (*b*) Cumulative distribution frequency plot of pooled DNA methylation levels for each treatment. Colours as in panel (*a*) and figure 1.

but changes in DNA methylation can also be the result of changes in cellular phenotype, which makes interpreting the physiological significance of changes in DNA methylation challenging [48]. For example, both developmental temperature and thermal acclimation have been shown to cause changes in the proportion of oxidative and glycolytic muscle fibres in fish [8,49]. Thus, the changes in DNA methylation that we observe could be either a cause or a consequence of changes in cell type.

Interspecific variation in genomic DNA methylation levels with body temperature has also been hypothesized to be the result of temperature effects on the rate of deamination of methylated cytosines [47]. This 'methylation-temperature-deamination hypothesis' posits that the relatively low methylation levels of endotherms and tropical fishes compared with ectotherms in cooler climates could be the result of reduced genome GC content in animals with higher body temperatures, and further suggests that this reduced GC content is the result of increased rates of deamination of methylated cytosines (causing increased mutation rate from C to T) at higher body temperatures [50]. In the context of this hypothesis, our observation of increased genomic DNA methylation levels in stickleback exposed to elevated temperatures could ultimately result in accelerated mutation rates over evolutionary time scales, due to the potential for a positive feedback between the increased rate of deamination due to elevated environmental temperatures and the increased genomic DNA methylation levels.

(b) Conserved differential methylation between temperature treatments

To determine whether there is a common core response of the epigenome to thermal change during both development and adult thermal acclimation, we summarized DNA methylation information across 100 bp windows, and identified 1206 differentially methylated regions (DMRs). In order to assess the potential functional significance of these DMRs we identified the nearest neighbour genes for each DMR (electronic supplementary material, table S4). We then performed functional (Gene Ontology; GO) enrichment analysis for annotated genes located within 2 kb of a DMR. There was no significant enrichment of GO categories for any treatment group.

Approximately 25% of the DMRs associated with variation in developmental temperature were also differentially methylated as part of the thermal acclimation response in adults (figure 4). We identified 172 DMRs that overlapped between stickleback that developed at cold temperatures and adult stickleback acclimated to cold temperatures, and 146 DMRs that overlapped between stickleback that developed at warm temperatures and adult stickleback that were acclimated to warm temperatures. Comparison of all DMRs identified 50 DMRs that were differentially methylated in response to all four treatments (figure 4), and only two of these DMRs showed different directions of response to temperature across treatment, while the other 48 regions all exhibited the same direction of change in DNA methylation level in all treatments (electronic supplementary material, table S5). These regions may represent candidates for a generalized response to thermal change. One of these common DMRs was located in the TNF receptor-associated factor 7 gene, an E3 ubiquitin ligase that has been previously identified as a target for natural selection with latitude or temperature in sea urchins [51] (electronic supplementary material, table S4). These data suggest that there is a common core response of the epigenome to thermal change, and highlights the possibility that developmentally induced variation in DNA methylation patterns could influence plasticity in adult acclimation responses.

The changes in methylation patterns that we detect in response to thermal change may point towards mechanisms underlying the transgenerational effects of temperature in stickleback [33–35,52,53], which have been suggested to

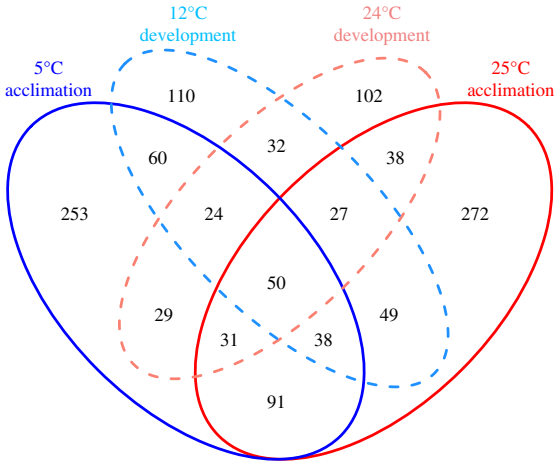

Figure 4. Venn diagram depicting the number of common and unique differentially methylated regions identified between stickleback that were developed at and acclimated to 18°C and stickleback that developed at 12°C (dashed light blue line) or at 24°C (dashed pink line) and then were acclimated to 18°C, or that developed at 18°C and were then acclimated to 5°C (solid dark blue line) or 25°C (solid red line).

buffer the short-term effects of climate warming. Whether the effects of temperature on stickleback methylation levels persist through multiple generations requires further investigation; however, the abundance of distinct differentially methylated loci in each treatment suggests that variability in environmental temperatures could contribute to the observed accumulation of epigenetic variation between stickleback morphotypes [54].

4. Conclusion

Understanding the mechanisms regulating the capacity of organisms to respond to environmental variation is a critical aspect of determining the impacts of environmental change on populations [1]. In this study we have demonstrated that altered temperature during development has prolonged effects on DNA methylation levels in an ectothermic vertebrate and that modifications to DNA methylation levels are also associated with the plastic adult acclimation response to environmental temperatures. In addition, we have demonstrated that the persistent effects of developmental plasticity on DNA methylation patterns affect regions of the genome where DNA methylation patterns are also modified during adult acclimation. These data illustrate the profound effect of temperature on DNA methylation patterns across multiple time scales, which has important implications for elucidating the underlying mechanisms that may modulate the capacity of organisms to cope with environmental change.

Ethics. All animal use was conducted under approved University of British Columbia animal care protocol A11-0372, and adhered to all guidelines and policies of the Canadian Council on Animal Care.

Data accessibility. Supplemental figures and tables supporting this article can be found in the electronic supplementary material. RRBS sequencing files can be downloaded from the NCBI sequence read archive (SRA study accession no. SRP116600).

Authors' contributions. D.C.H.M. and P.M.S. conceived and designed the study; D.C.H.M. executed the experiments and analysed the data. D.C.H.M. and P.M.S. drafted the manuscript.

Competing interests. We declare we have no competing interests.

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