Background *Acartia tonsa* is a wildly distributed abundant zooplankton. This species is greatly important from an ecological point of view; it forms a crucial link between primary producers and fish in the food chain and it has an essential part in biochemical cycling. Due to its vast distribution, individuals of this species can experience varying levels of environmental conditions like temperature, salinity and pH [1]. If CO_2 emission remains unchanged it is predicted that by 2100 global average ocean pH levels could fall to 7.67 as oceans are absorbing more and more atmospheric CO_2 [2]. Global ocean temperatures are also predicted to increase, at least by ~4°C by 2100 [3]. Given the large population size, short generation time and widespread nature of this copepod species adaptation to these changed environmental conditions is likely. However, it is important to understand the extent of evolvability of this species given its ecological role. Also, by investigating the molecular mechanisms of adaptation in this useful model organism, we can more deeply understand the underpinnings of adaptation to rapid environmental change in general. In this study, we focused on how DNA methylation can facilitate adaptation in response to high temperature and dissolved CO_2 conditions.

Individuals collected from wild populations were kept in common garden conditions for 3 generations. The copepods were then separated into four treatment groups: ambient (18°C, 400 ppm, "AA"), high dissolved CO_2 concentration (2000 ppm, "AH"), high temperature (22°C, "HA"), and both ("HH"). 18°C and 400 ppm were shown to be ideal for *A. tonsa* reproduction and survival, while hatching success and survival decreased at 2000 ppm and at 22°C, respectively [4,5]. In each treatment there were four replicates with ~3,000-5,000 individuals, which were reared under treatment conditions for 25 generations. DNA was extracted at generation 0 from individuals in the ambient treatment group, and from individuals from all treatment groups after 25 generations for reduced representation bisulfite sequencing (BS-Seq). To check the efficiency of the bisulfite conversion *E. coli* (unmethylated) DNA was added.

Bioinformatics Pipeline

Trimming and Mapping Trimming was done using Trimmomatic [6]. BS-Seq converts unmethylated cytosines into thymines, which reduces the GC content as well as the complexity of the sequences. This means that a special method of alignment is required for this type of sequence data. Also, two versions of the reference genome are needed: one where all cytosines are converted into thymines, and one where all guanines are converted into adenines. Reads were aligned to these two modified versions of the *A. tonsa* reference genome [7] using the flexible and time-efficient tool Bismark, which maps reads while simultaneously performing methylation calling [8]. Reads were mapped with the local alignment option bowtie2, which is memory efficient as it indexes the genome to keep the memory footprint small [9]. After mapping, methylation calls were extracted using the Bismark methylation extractor. As the bases at the beginning of the reads were generally more methylated than bases at other positions, these regions were trimmed off to avoid bias in our data due to some error in bisulfite conversion or sequencing. These steps yielded us a coverage file containing the positions and methylation rates of the nucleotides.

Testing for differential methylation The R package Methylkit was used to test for differential methylation [10]. First, we filtered out bases with very high coverage (>97.5) as these could be a result of technical errors like the presence of PCR duplicates. Then, we compared methylation in a pairwise manner; we calculated differential methylation between AA25 and AH25 and between AA25 and HA25 to determine the individual effects of CO_2 concentration and temperature on methylation patterns. We considered SNPs that were significantly differentially methylated (qvalue = 0.05), and where the methylation rate differed by at least 10%. To find out which genes these SNPs belong to, we used the "bedtools closest" program to search for positions in the *A. tonsa* annotation table [11].

Results The mapping success differed substantially between samples collected from different treatment groups, with AA generation 0 having the highest mapping rate of ~65%, while the average mapping rate for the rest of the groups was ~40%. There were no differences in the frequency distribution of methylation rates across SNPs between samples coming from different treatments. For all, most SNPs were unmethylated while the rest of the distribution followed a bell-curve shape where most SNPs had ~60% methylation rate. Individuals from group AA at generation 0 had a higher average methylation frequency per site than at generation 25 (ANOVA, $F_{1,6} = 6$, $p = 0.049$). However, this could be due to a higher mapping success of reads from the generation 0 samples. There was no difference in average methylation rate between samples of any of the treatment groups after 25 generations (ANOVA, $F_{3,12}=0.6761$, $p=0.58$). Despite no global changes, (i) the number of individual SNPs differentially methylated, (ii) the ratio of hyper- and hypomethylated sites and (iii) the genes with differentially methylated SNPs differed between samples from the high CO_2 and the high temperature treatment groups.

There were 30 significantly differentially methylated SNPs when ambient and high CO_2 treatment groups were compared, out of which 6 SNPs that were hyper- and 24 were hypomethylated. Most SNPs had a reduced methylation by 10-15% (see Figure 1). By searching in the annotation table, 3 SNPs were found within gene bodies. On the other hand, there were 105 significantly differentially methylated SNPs when ambient and high temperature treatment groups were compared, out of which 84 SNPs that were hyper- and 21 were hypomethylated. Most SNPs had an increased methylation by 15-20% (see Figure 1). By searching in the annotation table, 8 SNPs were found within gene bodies and 2 downstream.

Conclusion Epigenetic response to elevated temperature was clearly different from the response to elevated CO_2 concentration, both in magnitude and in the types of genes that were methylated. There were more than three times as many differentially methylated sites in samples from the high temperature than in the high CO_2 treatment group and in the former most sites were hypermethylated as opposed to in the latter. Since these sites were mostly in gene bodies, an upregulation of gene expression can be predicted in response to the temperature treatment while a general downregulation in response to the CO_2 treatment [12]. The genes that were differentially methylated also differed. In response to heat cyclin-D2, which is a positive regulator of G1/S cell cycle transition, was found to have differential methylation, as well as a gene that acts as an activator for GTPases that are involved in the regulation of cell division. Notably, methylation of genes controlling cell growth has been associated with heat stress before [13]. In response to high CO_2, a gene involved in repair of oxidative DNA damage was found to have decreased in methylation. It has been shown that high CO_2 concentration can lead to the formation of oxidative damage in vivo [14], and this SNP is located in the first intron of the gene body which has been associated with upregulation in response to methylation [15], thus this endonuclease might be upregulated to deal with increased DNA damage. One gene, deoxyuridine 5'-triphosphate nucleotidohydrolase, was found have increased methylation in response to both treatments. My hypothesis is that due to the increased level of bicarbonate available, more dUTP is synthesised in the cell de novo [16], but an excess of this molecule would increase DNA mutation rate as dUTP are accidentally incorporated instead of dTTPs, thus this enzyme that hydrolyses dUTP needs to be upregulated in response to CO_2 [17]. By hydrolysing dUTP and thus creating dUMP, this enzyme also provides the immediate precursor for dTTP synthesis (Uniprot accession A0A0B5E894) which is especially advantageous when the DNA needs to be repaired due to heat or oxidative damage.

In conclusion, differential methylation in response to CO_2 and heat treatment were not random with respect to function, indicating an adaptive advantage under these changed conditions. This could “buy time” for populations to gain beneficial mutations and adapt to the changed environmental conditions they’ll soon probably experience. In future studies a better method replacing multiple pairwise comparisons should be developed and once annotations for the *A. tonsa* genome improve SNPs in first introns and promoters should be identified as well.

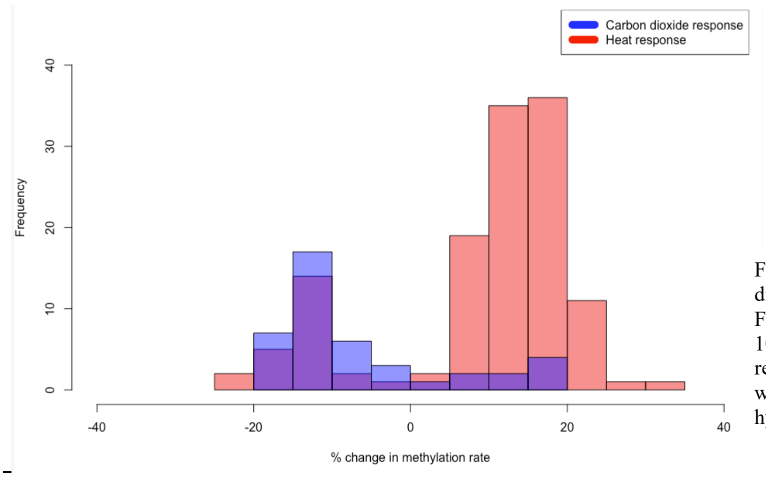

Figure 1. Frequency distribution of SNPs significantly differentially methylated in different treatment groups. For the purposes of this figure only, sites with less than 10% methylation rate difference were included. In response to heat, SNPs were largely hypermethylated while in response to CO_2 sites were largely hypomethylated.

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