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# Physiological Acclimatization in High-Latitude Zooplankton

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**Abstract**

How individual organisms adapt to non-optimal conditions through physiological acclimatization is central to predicting the consequences of unusual abiotic and biotic conditions such as those produced by marine heat waves. The Northeast Pacific, including the Gulf of Alaska experienced an extreme warming event (2014-2016, "The Blob") that affected all trophic levels leading to large-scale changes in the community. The marine copepod *Neocalanus flemingeri* is one key member of the subarctic Pacific pelagic ecosystem. During the spring phytoplankton bloom this copepod builds substantial lipid stores as it prepares for its non-feeding adult phase. A three-year comparison of gene expression profiles of copepods collected in Prince William Sound in the Gulf of Alaska between 2015 and 2017 included two high-temperature years (2015 and 2016) and one year with very low phytoplankton abundances (2016). The largest differences in gene expression were between high and low chlorophyll years, and not between warm and cool years. The observed gene expression patterns were indicative of physiological acclimatization. The predominant signal in 2016 was the down-regulation of genes involved in glycolysis and its incoming pathways, consistent with the modulation of metabolic rates in response to prolonged low food conditions. Despite the down-regulation of genes involved in metabolism, there was no evidence of suppression of protein synthesis based on gene expression or behavioral activity. Genes involved in muscle function were up-regulated, and the copepods were actively swimming and responsive to stimuli at collection. However, genes involved in fatty acid metabolism were down-regulated in 2016, suggesting reduced lipid accumulation.

## 1. Introduction

Marine heat waves have devastating effects on ecosystems leading to major die-offs of foundation species and ecological regime shifts that persist beyond the warm period (Arimitsu et al., 2021; Smale et al., 2019; Suryan et al., 2021). As marine heat waves (MHWs) increase in frequency and amplitude (Oliver et al. 2018), it becomes increasingly important to understand their impact on both individual organisms and entire biological communities. While anomalously warm temperatures may lead to high mortality in species living near their thermal limit (Smale et al., 2019), many observed ecosystem responses cannot be explained by differences in temperature. Predicting community responses and establishing causal effects and linkages among the multiple and cascading changes during MHWs are more difficult (Oliver et al., 2021; Suryan et al., 2021). The large warm temperature anomaly (the "Blob") that started in the North Pacific during the fall of 2013 exceeded historical variability in sea-surface temperatures recorded in the Gulf of Alaska (Litzow et al., 2020). It had devastating effects on fisheries and wildlife along the entire Gulf of Alaska, Bering Sea and even the southern California Current System (Peterson, Robert, & Bond, 2015). The weather-induced environmental changes cascaded through the entire food web with reports of low primary productivity, unusual harmful algal blooms, warm-water copepod species invading the sub-arctic, low larval fish recruitment and high mortality in salmon, sea birds, whales and sea lions (Auth, Daly, Brodeur, & Fisher, 2018; Gomez-Ocampo, Gaxiola-Castro, Durazo, & Beier, 2018; Nielsen et al., 2021; Piatt et al., 2020; Rogers, Wilson, Duffy‐Anderson, Kimmel, & Lamb, 2021; Suryan et al., 2021; Zhu et al., 2017). However, an analysis of abundance data of the pelagic community collected over a 20-year period in the Gulf of Alaska underscored the difficulty of understanding causal effects. While the study failed to identify significant correlations between sea-surface temperature (SST) and changes at the lower trophic levels (Litzow et al. 2020), an ecosystem-wide analysis not only detected shifts in the lower trophic levels, but concluded the community had not returned to pre-Blob conditions by 2018 (Arimitsu et al., 2021). These studies focused on abundances alone and provide no information on how organisms were responding

physiologically to environmental conditions that, in addition to temperature included many more factors including some hidden ones. An alternate approach is to query the organism as to their state of "health."

The study of organism-environment interactions in physiological ecology aims to establish adaptive capacity and niche breadth by elucidating how an organism adjusts its physiology to abiotic and biotic environmental stressors. A framework is emerging in this field that links known physiological responses to environmental stressors and the effect of acclimatization on Darwinian fitness (Sokolova, 2013). This requires studying organisms in the field, while applying knowledge gained from experimental studies on model species. For example, the cellular stress response (CSR) is a universal response among organisms that provides immediate protection against stress-induced macromolecular damage (Kültz, 2020). Well-studied physiologically, the CSR involves a minimal stress proteome (conserved proteins) whose expression is altered in response to the stress in order to repair and prevent damage, activate cell cycle checkpoint and reallocate energy (Gasch, 2003; Kültz, 2005). In contrast, organisms experiencing persistent non-optimal but non-lethal conditions will acclimatize by adjusting their physiology to maintain cellular homeostasis (Kültz, 2005, 2020). Physiological plasticity allows an organism to adjust to non-optimal conditions, however, at the expense of a decrease in fitness, i.e., lower reproductive success (Sokolova, 2013). Thus, acclimatization is typically energy-limited and affects both metabolism and innate immunity (Oomen, Hutchings, & Miller, 2017; Sokolova, 2013). However, homeostatic responses are also species-and stressor-specific (Kültz, 2005, 2020), and could offer an opportunity to elucidate causal linkages between the physiological response and the source of organismal stress. Physiological state and acclimatization have been characterized using high-throughput sequencing of mRNA (RNA-Seq) under experimental conditions in non-model organisms (Oomen et al., 2017). Building on such experimental studies, we examined physiological acclimatization in a key member of the subarctic Pacific plankton, the copepod *Neocalanus flemingeri* over a three-year period between

2015 and 2017. We used RNA-Seq to obtain gene expression profiles for individuals collected in the spring from their natural environment under contrasting conditions.

Our collection site, Prince William Sound (PWS) is a large fjord system that is inhabited by populations of lipid-rich copepods that diapause in the deep trenches of the sound (Cooney, 1986; Halverson, Bélanger, & Gay III, 2013; Kline Jr, 1999). In the spring, these small planktonic crustaceans are found in the upper 100 m where they feed on the spring phytoplankton bloom, accumulating lipids prior to entering diapause starting in late May (Cooney, 1986; Cooney, Coyle, Stockmar, & Stark, 2001; Coyle & Pinchuk, 2003, 2005; McKinstry & Campbell, 2018). Zooplankton biomass in the spring is typically dominated by three members of the genus *Neocalanus*, *N. flemingeri*, *N. plumchrus* and *N. cristatus* (Cooney, 1986; Cooney et al., 2001). *N. flemingeri*, the focus of this study is a filter feeder that consumes a wide range of single celled autotrophs and heterotrophs with a preferred food size greater than 20 μm (Dagg, Strom, & Liu, 2009; Liu, Dagg, Napp, & Sato, 2008). This species has non-feeding adults and depends on the spring phytoplankton bloom to store enough resources ("capital") to fuel both diapause and reproduction (Lenz & Roncalli, 2019; Roncalli, Cieslak, Hopcroft, & Lenz, 2020).

In the spring, chlorophyll *a* levels integrated between the surface and a depth of 50 meters can reach 100-200 mg per $m^2$ with major contributions from the copepod's preferred food size, large phytoplankton cells (> 20 μm) (McKinstry & Campbell, 2018; Strom, Olson, Macri, & Mordy, 2006). However, in 2016, at the peak of the Blob and the highest recorded surface temperatures, integrated chlorophyll *a* abundances were unusually low in PWS (McKinstry & Campbell, 2018; Strom et al., 2006), consistent with food-limited conditions (Liu & Hopcroft, 2006). *N. flemingeri* pre-adults (copepodite stage CV) were collected in early May in 2015, 2016 and 2017 during the annual spring oceanographic cruises of a long-term monitoring program (Coyle, Hermann, & Hopcroft, 2019; Sousa, Coyle, Barry, Weingartner, & Hopcroft, 2016). Using a comparative approach, we examined gene expression profiles to better understand physiological acclimatization under environmental conditions with different water temperatures, salinities and

spring chlorophyll *a* concentrations. Gene expression analysis was used to identify which biological processes, if any, were differentially regulated in individuals collected in the spring in three different years. The final goal was to examine phenotypic plasticity during a critical phase in the copepod's development, preparation for diapause, under contrasting environmental conditions.

## 2. Material and Methods

### *2.1 Sampling strategy and environmental data*

*Neocalanus flemingeri* pre-adults (copepodite stage V, CV) were collected during the May annual spring cruise of the Seward Line Long-term Observation Program (LTOP) (http://www.sfos.uaf.edu/sewardline/) from two stations in Prince William Sound (PWS) (Figure 1; PWS2: Lat 60° 32.1′, Long 147° 48.2′; PWSA: Lat 60° 49.3′, Long 147° 24′ ) during 2015 (May 7$^{th}$) , 2016 (May 1$^{st}$) and 2017 (May 3-4). Figure 1 also shows two additional PWS stations with environmental data (PWS1 and PWS3, see below) and two offshore Gulf of Alaska stations (GAK9 and GAK14). The latter were included in a previous transcriptomic study (Roncalli, Cieslak, Germano, Hopcroft, & Lenz, 2019). Zooplankton were collected using a QuadNet CalVET net towed vertically from 100 m depth to surface. Two of the four nets of the QuadNet had a mesh size of 150 µm and the others a mesh size of 53µm. For the transcriptomics samples, collections from the 53 µm mesh nets were removed prior to washing down the net and immediately diluted. *N. flemingeri* CVs were rapidly sorted under the microscope, preserved within 1hr of the tow into RNAlater Stabilization Reagent (QIAGEN) and frozen.

Environmental data collected as part of the spring collections (http://www.sfos.uaf.edu/sewardline/) were downloaded and analyzed for temperature, salinity, chlorophyll *a* and zooplankton abundances in western Prince William Sound (Hopcroft & Clarke-Hopcroft, 2019a, 2019b, 2019c) as summarized in Supplementary Table S1. Briefly, temperatures, measured using an SBE 911 + CTD, were averaged for the upper 50 meters for each year and then averaged across stations (PWS1, PWS2, PWS3, PWSA; Fig. 1). Size fractionated chlorophyll *a*

concentrations from discrete depths (0 – 50 m) were integrated and averaged across stations. Average *N. flemingeri* abundances (individuals per $m^3$) and biomass (g wet-weight $m^3$), and total zooplankton biomass were extracted from the QuadNet (150 μm mesh nets) zooplankton data for Prince William Sound.

*2.2 RNA extraction, gene library preparation and RNA-Seq*

Total RNA was extracted from individual CVs from the two stations (PWSA, PWS2) collected in 2015, 2016 and 2017 using QIAGEN RNeasy Plus Mini Kit (catalog # 74134) in combination with a Qiashredder column (catalog # 79654) following the instructions of the manufacturer and stored at -80ºC. Total RNA concentration and quality were checked using an Agilent Model 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Individuals with the highest quality and RNA yields (n=22 [2015: 6, 2016: 6 and 2017: 10]) were selected for RNA-Seq and shipped on dry ice to the University of Georgia Genomics Facility (dna.uga.edu). Extractions, gene library preparation and sequencing were completed in two batches with 2015 and 2016 samples in the first one; 2017 samples in the second. For each batch, double-stranded cDNA libraries were prepared from total RNA extracted using the Kapa Stranded mRNA-Seq kit (KK8420) following manufacturer's instructions. After enrichment of mRNA with oligo-dT beads samples were fragmented and reverse transcribed into double-stranded complementary DNA. Each sample was tagged with an indexed adapter and paired end sequenced (2015, 2016: PE150 bp; 2017: PE75 bp) using an Illumina NextSeq 500 instrument using High-Output Flow Cell. After quality assessment (FASTQC v1.0.0), any remaining Illumina adapters were removed and the first 9 bp were trimmed from each read using Trimmomatic (v. 0.36). Additionally, reads with low quality ("Phred" score < 30, matched pairs) and length < 50 bp were removed from each library. Each library resulted in 7 to 15 million high-quality reads per sample with an average of 9 million across all samples. The 2015 and 2016 RNA-Seq data (read length 150bp) were shortened to a final length of 75 bp prior to mapping to reduce methodological bias.

*2.3 Expression profiling and t-SNE clustering analysis*

Expression level was quantified, for each individual, by mapping each quality-filtered library against an existing *N. flemingeri* CV reference transcriptome (Roncalli et al., 2019) using kallisto software (default settings; v.0.43.1) (Bray, Pimentel, Melsted, & Pachter, 2016). For the counts generated by the kallisto mapping, relative expression levels were normalized by the TMM method (trimmed means of M values) followed by the RPKM method (reads per kilobase of transcript length per million reads) using EdgeR (Mortazavi, Williams, McCue, Schaeffer, & Wold, 2008; Robinson, McCarthy, & Smyth, 2010).

The dimensionality reduction method, t-distributed Stochastic Neighbor Embedding (t-SNE) (van der Maaten & Hinton, 2008) was applied to the relative expression data for the full set of transcripts (n=51,743) of all 22 individuals. Specifically, t-SNE was applied to the $\log_2$ transform of the relative expression data after adding a pseudo-count of 1 to the RPKM value for each transcript (i.e. $\text{Log}_2$[RPKM+1]) using the algorithm implemented in the R package Rtsne (v.0.13) (Krijthe, 2015). After testing several values for the controlling parameters, a perplexity parameter of 6 and a maximum number of iterations of 50,000 were settled upon, and the algorithm was run multiple times to ensure that the output was representative (Cieslak, Castelfranco, Roncalli, Lenz, & Hartline, 2020; van der Maaten, 2014; van der Maaten & Hinton, 2008).

*2.4 Identification of differentially expressed genes (DEGs) among years*

A generalized linear model (GLM) was used to identify interannual differences using the BioConductor package EdgeR (R v. 3.12.1) (Robinson et al., 2010). RNA-Seq libraries (n=22) with their relative transcript abundances from kallisto mapping were filtered to remove transcripts with very low expression levels (< 1 count per million) from all samples. As implemented by EdgeR, RNA-Seq libraries were normalized using the TMM method and checked for batch effects using limma (BioConductor v. 3.14, limma package version 3.50.0, and R v. 4.1.1) (Ritchie et al., 2015). After removal of low expression transcripts and TMM normalization, we tested the remaining 38,227 transcripts for differential gene expression across years using generalized linear model (GLM). This analysis was followed by three pairwise likelihood ratio tests: 1) 2015 vs 2016; 2)

2015 vs 2017 and 3) 2016 vs 2017. All p-values were adjusted using the Benjamini-Hochberg correction for false discovery rate (significance: p-value <0.05).

*2.5 Functional annotation and enrichment analysis*

Functional annotations of the DEGs were obtained by searching the *N. flemingeri* reference transcriptome that had been annotated against SwissProt, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Roncalli et al., 2019). Enrichment analysis on all DEGs identified by the GLM analysis was performed to identify the biological processes (GO) that were significantly over-represented among years. DEGs were compared against the 24,356 transcripts with GO terms in the reference transcriptome using the R-package topGO (v. 2.88.0) that employs a Fisher exact test with a Benjamini-Hochberg correction (p-value < 0.05; using the default algorithm weight01) (Alexa & Rahnenfuhrer, 2010).

Based on enrichment results, genes annotated with specific GO terms and KEGG enzymatic pathways were investigated in greater detail. Briefly, the annotated *N. flemingeri* reference transcriptome was searched for transcripts annotated with selected enriched GO terms (e.g., digestion [GO:0007586], muscle contraction [GO:0006936)]) and for GO terms at a higher level of ontology (e.g. fatty acid (FA) biosynthetic process [GO:0006631]). In addition, we searched for transcripts annotated to the GO term cellular response to stress [GO:0033554]. Similarly, the reference transcriptome was searched for transcripts annotated with the KEGG enzyme commission numbers (EC) involved in metabolic pathways (glycolysis, glycolysis incoming pathways, tricarboxylic acid cycle) that were related to enriched processes, using the fruit fly *Drosophila melanogaster* as the reference organism. The DEGs were then searched for transcripts that resulted from these searches. Relative expression of these transcripts is shown in heatmaps as z-scores calculated from the log-transformed expression levels ($Log_2$[RPKM+1]). These searches often identified multiple transcripts with the same annotation (EC = enzyme commission number); we have included the relative expression of all of these transcripts in the heatmaps if e-values were ≤ $10^{-20}$.

## 3. Results

### *3.1 Ecological context: Interannual conditions in Prince William Sound*

The three study years represented contrasting environmental conditions in Prince William Sound in terms of water temperatures, chlorophyll *a* (an indicator for phytoplankton) availability and *N. flemingeri* abundances (Figure 2). The first two years, 2015 and 2016 were years of warm conditions with average temperatures in the upper 50 m reaching 6.3 and 6.5ºC, respectively (Figure 2A). The following year (2017) was much cooler with temperatures averaging 5.5ºC. Average salinities showed the inverse pattern being highest in 2017 (Figure 2A). Chlorophyll *a* of small and large cells of phytoplankton differed among years (Figure 2B). The interannual variation of large-cell chlorophyll *a* was particularly pronounced and exceeded two orders of magnitude between 2015 and 2016, with intermediate, but low, levels for both large and small cells in 2017. Despite these large differences in chlorophyll *a*, total zooplankton biomass was similar across years (Figure 2C). It showed a slight trend of increasing biomass between 2015 and 2017, but this trend was small compared with observed variability among stations. In contrast, the relative contribution of *N. flemingeri* to total biomass in PWS differed greatly ranging from >20% in 2016 to less than 1% in 2017, and this was reflected in the abundance data, which ranged between 6 ind $m^{-3}$ (2017) and nearly 100 ind $m^{-3}$ (2016; Figures 2C, 2D). In summary, conditions in 2016 were characterized by the warmest temperatures, the highest *N. flemingeri* abundances and the lowest chlorophyll *a* concentrations compared with the other two flanking years. While temperature and salinity in 2017 had returned to pre-Blob conditions and average chlorophyll *a* was slightly higher, *N. flemingeri* abundances in 2017 were very low and contributed little biomass to the zooplankton community.

### *3.2 Transcriptional phenotypes varied among years*

The t-SNE analysis, which is based on the relative expression of all genes (n=51,743) aggregated the CV expression profiles into distinct transcriptional phenotypes. The 2015 and 2016 individuals separated into two groups in the t-SNE plot (Figure 3). The expression profiles of the

2017 individuals were more variable as shown in the plot with eight individuals clearly separated from both the 2015 and 2016 individuals and the remaining two individuals, collected at station PWS2, clustering with the 2016 individuals (Figure 3).

*3.3 Large interannual differences in gene expression between 2015 and 2016*

Large interannual transcriptional differences among years were confirmed in the statistical analysis, which identified 6,327 differentially expressed genes (DEGs) across the three years (Supplementary Figure S1). The largest number of DEGs was found between the 2015 and 2016 individuals based on *a posteriori* pair-wise comparisons (> 4,000 DEGs, Supplementary Table S2). Gene expression differences were characterized by the over-representation of genes annotated to metabolic and multicellular organismal processes and muscle contraction (Table 1). Among the DEGs, seven GO terms were enriched and related to carbohydrate (n=3) and fatty acid (n=1) metabolism, muscle development and function (n=2) and digestion (n=1). The large difference in expression between 2015 and 2016 was confirmed by a weighted gene network correlation analysis (WGCNA), which identified a set of more than 1,000 DEGs correlated with large-cell and total chlorophyll *a* concentrations (Supplementary Figure S2, Table S3).

*3.4 Metabolic processes: drivers of transcriptional differences among years*

*3.4.1 Glycolysis and "incoming" metabolic pathways were down-regulated during 2016*

Overall, transcripts annotated to metabolic pathways involved in exergonic (energy-producing) processes defined gene expression differences among years. Sixty-four percent (93 out of 146) of genes in the reference transcriptome annotated as enzymes involved in the glycolysis pathway were differentially regulated across the years. The expression pattern was consistent across all glycolytic enzymes (*hexokinase*, *glucosephosphate isomerase*, *phosphofructokinase*, *fructose-bisphosphate aldolase*, *triosephosphate isomerase*, *glyceraldehyde 3-phosphate dehydrogenase*, *phosphoglycerate kinase*, *phosphoglycerate mutase*, *enolase* and *pyruvate kinase*) with DEGs significantly down-regulated in 2016 compared with both 2015 and 2017 individuals (Figure 4). In 2017, the relative expression of these genes differed between the two stations with overall lower

expression in PWS2 individuals then PWSA. This is consistent with the t-SNE results (Figure 3) that placed two individuals from PWS2 close to the 2016 individuals.

Carbon substrates enter glycolysis through additional metabolic pathways that feed intermediate compounds into glycolysis ("incoming" pathways; Figure 5). The initial steps of glycolysis are supported by enzymes associated with glycogen and galactose metabolism, followed by glycosaminoglycan and nucleotide sugar metabolism related enzymes that contribute to the intermediate phases. In *N. flemingeri*, nearly 45% (36 out of 82) of the genes in the reference transcriptome annotated to enzymes involved in these "incoming" metabolic pathways were differentially expressed among the three years. Overall, relative expression of DEGs involved in these metabolic pathways was significantly lower during 2016 compared with 2015 and 2017 individuals (Figure 5). In 2017, the relative expression of the genes annotated to glycogenolysis differed between the two stations with consistently higher expression in PWSA individuals then PWS2. A number of these DEGs (13/36) were among the genes positively correlated with chlorophyll *a* by WGCNA (Supplementary Figure S2).

*3.4.2 Tricarboxylic acid cycle (TCA) was down-regulated in 2016*

Twenty-four percent (6 of 25 genes) of the genes in the reference transcriptome involved in the tricarboxylic acid cycle showed significant interannual differences. DEGs included the enzymes *citrate synthase, aconitase*, *isocitrate dehydrogenase*, *succinate dehydrogenase*, and *fumarate hydratase*. In general, gene expression was lower in 2016 compared with the 2015 and 2017 individuals (Figure 6).

Noticeably absent from the DEGs were genes involved in ß-oxidation, an alternative pathway into the TCA cycle via Acetyl-CoA. None of the genes encoding the four core enzymes involved in ß-oxidation (*acyl-CoA dehydrogenase*, *enoyl-CoA hydratase*, *hydroxy acyl-CoA dehydrogenase*, *ketoacyl-CoA thiolase*) were differentially expressed among the years (data not shown).

*3.4.3 Fatty acid metabolism was down-regulated during 2016 and 2017*

Entry into diapause requires lipid accumulation to support the diapause phase and fuel reproduction. Fatty acid biosynthesis, parent GO term of the enriched fatty acid elongation process (GO:0034626), included 43 DEGs (Roncalli et al. Dryad publication). The general expression pattern of these genes was down-regulation in 2016 individuals compared with both 2015 and 2017 (Figure 7). Among the transcripts, 14 were annotated as *fatty acid (FA) elongases* (*ELOV4* & *ELOV6).* These were down-regulated in 2016 in comparison with 2015 with most of them having intermediate expression in 2017 (Figure 7). Among the other DEGs involved in lipid accumulation there were several *reductases*, *desaturases* and *fatty acyl coA synthase*. These genes were more highly expressed in 2015 individuals than in 2016. However, compared with the ELOVs, the expression of these transcripts was more variable across stations in 2015 and 2017. Lastly, several *fatty acid binding proteins*, transporters associated with FA biosynthesis, were differentially expressed with low expression in 2016 compared with higher but variable expression in 2015 and 2017 individuals (Figure 7). Many of the DEGs involved in fatty acid metabolism were also among the genes that were positively correlated with chlorophyll *a* by WGCNA (Supplementary Figure S2).

*3.5 Expression patterns were inconsistent with a cellular or thermal stress response*

In 2015, offshore Gulf of Alaska individuals ("Gulf", Figure 8A) showed high expression of genes involved in the cellular stress response (Roncalli et al., 2019). However, in the comparison across the three years within PWS, only a small number of DEGs were annotated to response to stress. Relative expression of antioxidant defense biomarkers, such as *superoxide dismutase* (*SOD*), *catalases*, *glutathione S-transferases* (*GST*), and *metallothioneins* were low in all years, in contrast to their relative expression in individuals collected offshore in the Gulf of Alaska during 2015 (Figure 8A). Heat shock proteins (HSPs) are molecular chaperones that stabilize proteins during thermal stress and are typically up-regulated during thermal stress (Feder & Hofmann, 1999; Hofmann & Todgham, 2010). Of the 6,327 DEGs (GLM), we found five heat shock proteins (HSPs) with only two genes up-regulated for *N. flemingeri* collected during the warmest year

(2016) compared with the other two years. These two HSPs, annotated as a constitutive *HSP70* (*Hsc 70-4*, blast E-value=0) and a small *HSP27*, showed a 2-fold difference in expression between 2016 and 2015. The remaining HSPs, annotated as *HSP82* and *HSP83* (two transcripts) were more highly expressed in 2017 compared with 2015 individuals, but not compared with 2016.

*3.6 Genes annotated to digestion and muscle contraction were differentially expressed*

Digestion was enriched with a total of 52 DEGs annotated with this GO term. Differences in expression included 24 DEGs annotated as *chymotrypsins* and *trypsins* that were more highly expressed in 2015 than in 2016 individuals (Figure 8B). Previously published data for "Gulf" individuals show that these 24 DEGs were also down-regulated in offshore *N. flemingeri* in 2015 in comparison with PWS. A smaller set of 14 DEGs were more highly expressed in 2016 and 2017, as well as offshore individuals than in PWS 2015 individuals.

Two processes involved in muscle function were overrepresented among the DEGs (Table 1). Seventy-five percent of the DEGs (n=63) were annotated as *myosins* and were highly expressed in 2016 compared with 2015 and 2017 individuals (Table 1, Figure 8C). Annotation of these *myosins* suggest involvement in muscle function including development and locomotor activity. A smaller number of DEGs (n=16), mostly annotated as *troponins*, were mostly up-regulated in 2017 individuals (Figure 8C). The expression of these genes were highly variable in offshore "Gulf" individuals. As with the incoming pathways to glycolysis and fatty acid metabolism, a number of DEGs involved in digestion (46/52) and muscle function (55/63) were identified by WGCNA as correlated with chlorophyll *a* concentrations (Supplementary Figure S2).

## 4. Discussion

*4.1 Overview*

The serendipitous timing of this study has produced an environmental RNA-Seq dataset for *Neocalanus flemingeri* that spans three critical years. It coincided with two years of high sea-surface temperatures followed by a cooler year marking the end of the "Blob" (Peterson, Bond, &

Robert, 2016; Suryan et al., 2021). Large-scale effects on ecosystems were reported throughout the Northeast Pacific, and included mass mortality events, low recruitment for some species and community regime shifts in the Gulf of Alaska (Arimitsu et al., 2021; Piatt et al., 2020; Suryan et al., 2021). However, the complexity of the response cannot be explained by direct effects of temperature alone: species abundances at the lower trophic levels of the pelagic community could not be simply linked with sea-surface temperature (Litzow et al., 2020). We also found that the largest number of differentially expressed genes in *N. flemingeri* were not correlated with temperature but with chlorophyll *a* levels. Furthermore, expression differences of genes regulating energetic metabolism suggest responses to food availability. Thus, while both temperature and food are drivers of copepod growth, development and fecundity (Batchelder et al., 2013; Campbell, Wagner, Teegarden, Boudreau, & Durbin, 2001; Liu & Hopcroft, 2006), the large difference in gene expression between 2015 and 2016 individuals was more likely to be related to chlorophyll *a* than temperature.

Environmental transcriptomics of planktonic organisms has contributed new insights into niche separation and led to new hypotheses to explain rapid changes in abundances (Alexander, Jenkins, Rynearson, & Dyhrman, 2015; Mojib, Thimma, Kumaran, Sougrat, & Irigoien, 2017). *N. flemingeri* is well-suited for environmental transcriptomics: it has a single generation per year with an annual phenology that is predictable and synchronized with the annual spring phytoplankton bloom (Cooney, 1986; Cooney et al., 2001; Coyle et al., 2019; Coyle & Pinchuk, 2003). In early May, *N. flemingeri* pre-adults (stage CV) are abundant in the upper mixed layer with peak numbers between the surface and 40 meters (Coyle & Pinchuk, 2005), where they feed preferentially on large phytoplankton cells and ciliates (Dagg et al., 2009; Liu et al., 2008). Although abundances varied among years, the majority (>90%) of *N. flemingeri* were in the stage CV in early May in PWS (Hopcroft & Clarke-Hopcroft, 2019c) preparing for their annual ontogenetic migration to depth where they molt into adults, mate and diapause (Cooney et al., 2001; Miller & Clemons, 1988). Thus, *N. flemingeri* individuals collected during the three years were at a similar stage in

their life cycle and year-to-year differences in gene expression are likely to reflect organismal acclimatization to ambient environmental conditions.

*4.2 Adaptation to low food conditions*

Organisms inhabiting highly seasonal environments that include low/no food conditions possess physiological adaptations like diapause that enhance their ability to withstand food deprivation. Preparation for predictable low food periods involves storage of energy resources to enable survival during the non-feeding period that may last many months. A lipid accumulation phase is widespread among arthropods, including copepods that undergo a post-embryonic diapause (Baumgartner & Tarrant, 2017; Denlinger & Armbruster, 2014). The phase is synchronized to a period of abundant food, i.e., the spring phytoplankton bloom to ensure adequate provisioning for the non-feeding period. While many copepods resume feeding after diapause, *N. flemingeri* does not: adults are non-feeding, and growth and lipid accumulation for both diapause and reproduction are limited to approximately 1/3 of their lifespan (Lenz & Roncalli, 2019).

A central question is what happens if food resources are in short supply during the growth and lipid accumulation phase. While the annual spring phytoplankton bloom characterizes the Gulf of Alaska, food conditions can be unpredictable at the scale of an individual copepod given spatial, seasonal and year-to-year variability (Mackas & Coyle, 2005; Waite & Mueter, 2013). Thus, encountering low food conditions is not unusual for *N. flemingeri* during the spring (Coyle et al., 2019; Dagg et al., 2009; Liu & Hopcroft, 2006; Mackas & Coyle, 2005). Mackas and Coyle (2005) hypothesized that the copepod population in the Gulf of Alaska is comprised of winners that are advected into resource-rich regions like large eddies and nearshore areas, and losers that find themselves in nutrient poor conditions and are unlikely to survive. However, such a dichotomy fails to capture the complexity and temporal variability of food conditions encountered by individual copepods. Our results suggest that physiological acclimatization plays a significant role in the copepod's adaptive capacity and resilience to non-optimal food conditions.

*4.3 Food conditions in Prince William Sound (PWS)*

Prince William Sound is characterized by earlier phytoplankton blooms and higher chlorophyll *a* levels than oceanic regions in the Gulf of Alaska (Strom et al., 2006; Waite & Mueter, 2013). However, year-to-year differences in chlorophyll *a* are common throughout the region. During 2015 and 2016 early May chlorophyll *a* measurements were, respectively, at the highest and the lowest levels observed within the western PWS region between 2012 and 2017 (Hopcroft & Clarke-Hopcroft, 2019a). During 2016 maximum chlorophyll *a* concentrations between 0 and 50 m were mostly below 0.5 µg per liter while in 2015 they exceeded 4.5 µg per liter. Furthermore, chlorophyll *a* levels of large cells, the copepod's preferred food size (Dagg et al., 2009), differed by two orders of magnitude. One organismal response to food deprivation is the down-regulation of digestive enzymes and their gene expression (Wang, Hung, & Randall, 2006), which has been reported in copepods (Freese, Søreide, & Niehoff, 2016; Hassett & Landry, 1990; Mayzaud, Roche-Mayzaud, & Razouls, 1992). Thus, not surprisingly, genes involved in digestion were differentially expressed in *N. flemingeri* under low chlorophyll *a* conditions. Furthermore, based on shipboard grazing experiments, *N. flemingeri*'s feeding rates would have ranged from maximum in PWS in 2015 to low in 2016 and 2017 at the measured chlorophyll *a* levels (Dagg et al., 2009). Biomass of another preferred food, heterotrophic ciliates and other microzooplankton (Dagg et al., 2009), was also very low in 2016 and 2017 in comparison with 2015 and earlier years (Suryan et al., 2021). Thus, food limitation would have been predicted for *N. flemingeri* based on ambient chlorophyll *a* concentrations and microzooplankton abundances in PWS during May 2016 (Dagg et al., 2009; Liu et al., 2008), however, no studies have investigated the copepod's physiological response to these environmental conditions.

*4.4 Expression patterns suggest low metabolic rates, instead of dependence on stored energy during 2016*

Very low expression of genes involved in metabolic processes concerned with energy production (glycolysis and pathways feeding into glycolysis) characterized the *N. flemingeri* CV individuals in 2016. Down-regulation of metabolic genes is associated with reduced metabolic rates

in both vertebrates and invertebrates during prolonged periods of food deprivation (Wang et al., 2006). The large contrast in the expression patterns of these genes between 2015 and 2016 in Prince William Sound is summarized diagrammatically in Figure 9. During 2017 and offshore in the Gulf of Alaska, expression of these genes was variable but in general intermediate between 2015 and 2016. The down-regulation of the entire glycolysis pathway and most glycolysis in-coming pathways for 2016 suggests that the CVs decreased their metabolic rates to acclimatize to the unusually low food conditions that year.

In the initial phases of food deprivation, organisms maintain their metabolic rate and homeostasis by switching to other sources of energy (Sokolova, 2013; Wang et al., 2006). The observed differences in gene expression are inconsistent with this type of response. Carbohydrate is the main fuel source and is the first that is affected during low food conditions as shown in starvation experiments in crustaceans and the fruit fly *Drosophila melanogaster* (Arrese & Soulages, 2010). Genes annotated to enzymes involved in glycogen metabolism were down-regulated in 2016. A switch to lipids as an energy source, another expected response to low food conditions, is characterized by the up-regulation of the ß-oxidation pathway that feeds into the TCA cycle. This response was absent in PWS individuals, but was reported in offshore *N. flemingeri* CV individuals during 2015 (Figure 9): all genes annotated to ß-oxidation were significantly up-regulated in comparison with PWS individuals (Roncalli et al., 2019). The low expression of genes involved in fatty acid catabolism via ß-oxidation suggests that the CVs in PWS did not rely on stored lipids to meet their energetic needs during low food conditions. However, a reduction in metabolism necessitates the down-regulation of other energy-requiring processes (Wang et al., 2006). In *N. flemingeri*, genes involved in lipid biosynthesis and storage were down-regulated during 2016, which is consistent with reduced lipid accumulation and probably contributed to relatively low total fecundity of the females in 2016 compared with other years (Hopcroft & Lenz, 2021; Slater, 2004).

## 5. Concluding remarks

Marine heat waves provide opportunities to study organismal acclimatization to contrasting and extreme environmental conditions, such as those represented by the three-year study period in the Gulf of Alaska. The goal was to investigate adaptive capacity and resilience under these contrasting conditions by generating gene expression profiles of a key planktonic organism. Low food conditions are not unusual for zooplankton in general and in some species can lead to rapid changes in abundances. In contrast, others, like *N. flemingeri* are resilient, however, the basis their adaptive capacity during their active phase is poorly understood. Gene expression patterns observed in *N. flemingeri* were consistent with a long-term response to low food conditions during 2016 and to a lesser extent during 2017. In addition, gene expression profiles suggest a decrease in metabolic rates in combination with limited use of stored resources during low food conditions. Such a response to low food would contribute to the copepod's ability to retain stored lipids, which are required for diapause and reproduction. However, food resources were very low during 2016, and whether conditions came to close to a tipping point for the survival of the local Prince William Sound population in this year remains an unanswered question.

## Data availability

The metadata generated and analyzed during the current study are available through Alaska Ocean Observing System (AOOS) Research Workspace and the Biological & Chemical Oceanography Data Management Office (BCO-DMO) repository under the Project: *Neocalanus* Gulf of Alaska [https://www.bco-dmo.org/project/542182]. The raw RNA-Seq data for all the libraries are available through National Center of Biotechnology Information (NCBI; BioProject: PRJNA496596). A companion data publication in Dryad (doi:10.5061/dryad.kh1893273) includes output from the statistical analysis and the annotation file for the reference transcriptome deposited at NCBI (GenBank accession: GLBH0000000.1).

## Benefits shared statement

The work provides the benefit of public access to hard-to-obtain well-vetted RNA-Seq data, especially laying a baseline against which future changes can be measured. Methodological approaches used provide a model for effective application of the tools of environmental transcriptomics to understanding marine organisms and predicting their future courses. The study supports research priorities of the United Nations Decade of Ocean Science for Sustainable Development (2021-2030) initiative. Specifically, for the Gulf of Alaska, the study contributes to an understanding of resilience in zooplankton upon which this highly-productive ecosystem depends. It integrates with and benefits the Northern Gulf of Alaska Long Term Ecological Research Program.

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**Author Contributions**

VR, PHL and RRH conceived the sampling design; JN, VR, AMC, MCC and PHL analyzed the data; JN, VR and PHL interpreted the data, wrote and reviewed the manuscript. All authors were involved in editing the manuscript and approved the final version.

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## Supplemental Information for:

# Physiological Acclimatization in High-Latitude Zooplankton

Vittoria Roncalli, Jeanette Niestroy, Matthew C. Cieslak, Ann M. Castelfranco, Russell R. Hopcroft, and Petra H. Lenz

## Table of Contents:



**Table S1.** Summary of environmental data for western Prince William Sound, 2015-2017. Data are publicly available (Hopcroft and Clarke-Hopcroft, 2019a, b, c).

| Year | Sampling date | Station | Temperature (ºC) | Chlorophyll *a* | Zooplankton (QuadNet CalVET) | *N. flemingeri* RNA-Seq |
|---|---|---|---|---|---|---|
| 2015 | May 7 | PWS1 | X | X | X | |
| | May 7 | PWS2 | X | X | X | X |
| | May 8 | PWS3 | X | X | | |
| | May 8 | PWSA | X | X | | X |
| 2016 | May 1 | PWS1 | X | X | X | |
| | May 1 | PWS2 | X | X* | X | X |
| | May 2 | PWS3 | X | X | | |
| | May 1 | PWSA | | | | X |
| 2017 | May 4 | PWS1 | X | X | X | |
| | May 4 | PWS2 | X | X | X | X |
| | May 4 | PWS3 | X | X | X | |
| | May 3 | PWSA | X | X | | X |

* Chlorophyll *a* was measured twice at station PWS2 in 2016, the second measurement was made on May 2.

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**Table S2. Summary of differential gene expression analysis.** Differentially expressed genes (DEGs) were identified using general linear model (GLM) followed by pairwise likelihood ratio tests (FDR; p-value ≤ 0.05) between 2015, 2016 and 2017 individuals. For each pairwise likelihood test, the number of total DEGs and relative expression for each year. For each paired comparison the number of genes that were up-regulated in a particular year is noted.

| | **DEGs** | **Expression is up-regulated in:** | | |
|---|---|---|---|---|
| | | 2015 | 2016 | 2017 |
| General linearized model (GLM) | 6,327 | | | |
| Likelihood ratio test: | | | | |
| 2015 vs 2016 | 4,312 | 2,969 | 1,343 | |
| 2016 vs 2017 | 3,400 | | 2,330 | 1,070 |
| 2015 vs 2017 | 2,371 | 1,423 | | 948 |

**Table S3. Environmental variable used in the weighted-gene network correlation analysis (WGCNA) (Figure S2).** Chlorophyll *a* concentrations, measured for two size fractions, < 20 µm (Chl*a* [sml]) and > 20 µm (Chl*a* [lge]) at 5 depths were averaged. Total chlorophyll *a* (Chl*a* [total]) is the sum of the large and small size fractions. Temperature and salinity were measured using a CTD and averaged from 0 to 100 m. Source of data: see Table S1.

| **Variable** | **2015 PWS2** | **2015 PWSA** | **2016 PWS2** | **2016 PWSA*** | **2017 PWS2** | **2017 PWSA** |
|---|---|---|---|---|---|---|
| **Chl*a* (lge)** | 1.04 | 2.16 | 0.01 | 0.02* | 0.01 | 0.07 |
| **Chl*a* (sml)** | 1.29 | 1.14 | 0.24 | 0.48* | 0.08 | 0.21 |
| **Chl*a* (total)** | 2.33 | 3.30 | 0.25 | 0.50* | 0.09 | 0.28 |
| **Temperature** | 6.05 | 6.44 | 6.38 | 6.45* | 5.32 | 5.47 |
| **Salinity** | 31.0 | 30.9 | 30.4 | 30.7* | 31.8 | 31.7 |

* No chlorophyll *a*, salinity or temperature measurements were available for PWSA/2016. Measurements listed (and used in WGCNA) were made at PWS3.

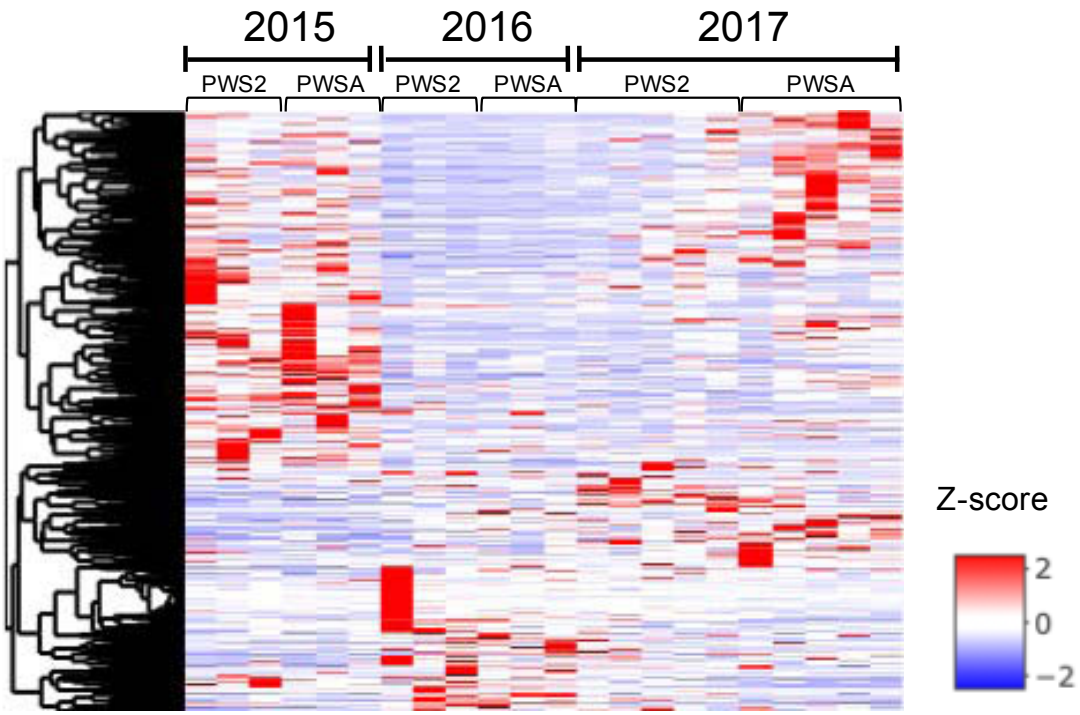


**Figure S1. Heatmap showing patterns of gene expression in *Neocalanus flemingeri* CVs from two stations (PWS2, PWSA) in 2015, 2016 and 2017.** Relative expression shown as z-scores of differentially expressed genes (n = 6,327). Each column indicates an individual CV aggregated by station and by year as shown by brackets on top. Relative expression is color coded by the magnitude of differential expression between expression level for each individual and mean expression across all individuals in $\log_2$(RPKM+1) normalized by the variance (scale bottom left). Genes were ordered by similarity of expression pattern as shown by the dendrogram (left). DEGs were identified by GLM test with $p \leq 0.05$ after FDR correction.

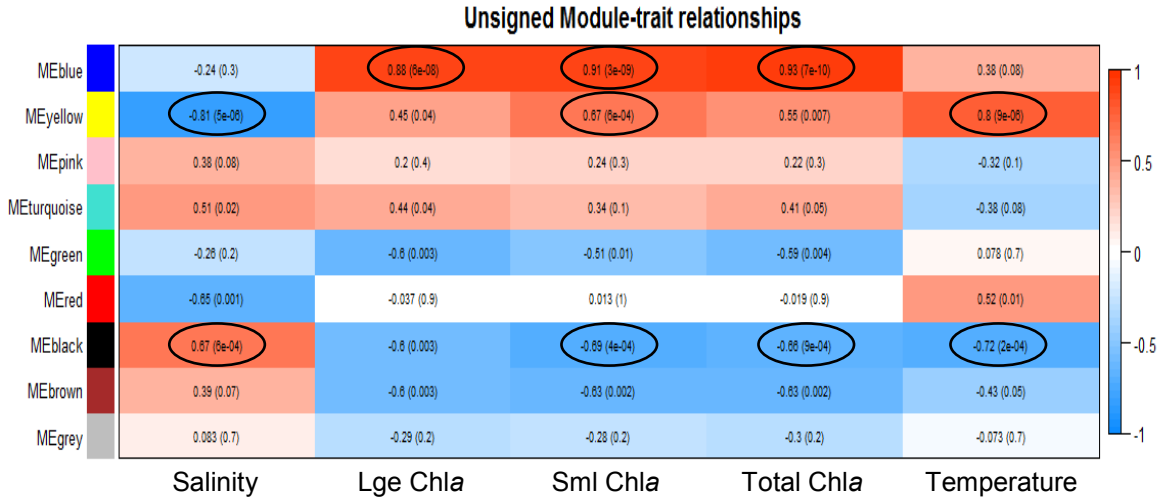


**Figure S2. Weighted-gene network correlation analysis (WGCNA) of differentially expressed genes (DEGs) among 2015, 2016 and 2017.** Each color module includes sets of differentially expressed genes (DEGs) with similar expression patterns and their correlation coefficients based on different variables (year, salinity, large-cell chlorophyll a, total chlorophyll a, temperature and station/year). Environmental variables for the analysis: chlorophyll *a*, large cells (lge), small cells (sml) and total (µg/l average: 0-50 m), salinity (PSU) and temperature (ºC) (Table S3). Significant correlations for chlorophyll *a*, salinity and temperature (> ± 0.65) are circled. Blue module: 1351 DEGs positively correlated with chlorophyll *a*. Yellow module: 358 DEGs positively correlated with temperature and small cell chlorophyll *a*, and negatively correlated with salinity. Black module: 217 DEGs positively correlated with salinity and negatively correlated with temperature and small cell and total chlorophyll *a*. Lists of DEGs in each module are available in the companion Dryad publication. The blue module included DEGs annotated to KEGG enzymes and GO term of genes shown in Figures 4 (n=1), 5 (n=7), 7 (n=16) and 8 (n=57).

*Methods:* Patterns of differential gene expression among samples were further explored using weighted correlation network analysis (WGCNA) (Langfelder and Horvath 2008; 2012). The WGCNA analysis was performed on the log-transformed ($Log_2$[RPKM+1]) gene expression of all DEGs (n=6,319) identified by the generalized linear model (GLM, EdgeR, BioConductor package) applied to the RNA-Seq libraries after normalization by the TMM and RPKM methods and removal of low expression transcripts. The WGCNA analysis used an unsigned network type (networkType= "unsigned" and TOMType="signed") with a soft threshold power of 8 and minimum module size of 100 transcripts. The Pearson correlations between the module eigengenes (a weighted average of the module expression profiles) and measured environmental variables: chlorophyll a, salinity and temperature were computed. The results of the correlation analysis were visualized as a heatmap.

References: Langfelder P and Horvath S, (2008) WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics*, 9:559. doi:10.1186/1471-2105-9-559; Langfelder, P., and Horvath, S. (2012). Fast R Functions for Robust Correlations and Hierarchical Clustering. *Journal of Statistical Software*, *46*(11), 1–17. https://doi.org/10.18637/jss.v046.i11