Functional Ecology

British Ecological Society

Functional Ecology 2012, **26**, 144–155 doi: 10.1111/j.1365-2435.2011.01924.x

Transcriptome profiles link environmental variation and physiological response of *Mytilus californianus* between Pacific tides

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Summary

1. The marine intertidal zone is characterized by large variation in temperature, pH, dissolved oxygen and the supply of nutrients and food on seasonal and daily time scales. These oceanic fluctuations drive ecological processes such as recruitment, competition and consumer–prey interactions largely via physiological mechanisms. Thus, to understand coastal ecosystem dynamics and responses to climate change, it is crucial to understand these mechanisms.

2. Here we utilize transcriptome analysis of the physiological response of the mussel *Mytilus californianus* at different spatial scales to gain insight into these mechanisms. We used mussels inhabiting different vertical locations within Strawberry Hill on Cape Perpetua, OR and Boiler Bay on Cape Foulweather, OR to study inter- and intra-site variation of gene expression.

3. The results highlight two distinct gene expression signatures related to the cycling of metabolic activity and perturbations to cellular homeostasis. Intermediate spatial scales show a strong influence of oceanographical differences in food and stress environments between sites separated by *c*. 65 km.

4. Together, these new insights into environmental control of gene expression may allow understanding of important physiological drivers within and across populations.

Key-words: biogeographical distribution, DNA microarray, ecological genomics, environmental stress, gene expression, mussels, *Mytilus californianus*, spatial variation

Introduction

In mussel communities along the Oregon coast, basal gene expression patterns as well as physiological responses are likely set by the interplay of multiple, complex abiotic factors. Temperature, and more specifically body temperature, and food availability have been shown to be two of the most important determinants of survival, growth and reproduction (e.g. Halpin *et al*. 2004, Helmuth *et al*. 2006; Menge, Chan & Lubchenco 2008). Understanding the underlying mechanisms by which body temperature and food availability drive organismal responses and physiological performance is becoming increasingly imperative as climate change alters habitat temperature (Somero 2010). Until recently, we lacked the ability to examine physiological responses at a level of complexity commensurate with that of the changing environment of an organism. Previous studies examining the mechanistic links between the ecology of an organism and its cellular responses have been largely observational, and are limited in scope to a small number of abiotic or biotic factors, and the resulting changes of single bioindicators, such as heat-shock proteins (Roberts, Hofmann & Somero 1997; Osovitz & Hofmann 2005; Sagarin & Somero 2006), or relatively basic proxies for physiological state, such as growth rate (Levinton & Monahan 1983; Yamahira & Conover 2002), body size (Roy & Martien 2001), reproductive output (Lonsdale & Levinton 1985; Leslie *et al*. 2005), and mortality (Ebert *et al*. 1999; Zippay & Hofmann 2010). Now, genome-wide analyses performed within the framework of an ecological context allow us to examine the interplay of multiple drivers and organismal responses (for reviews, see Hofmann & Place 2007; Aubin-Horth, Letcher & Hofmann 2009).

Previous analyses of variation in transcript levels have highlighted the potential application of ecological genomics to understand physiological responses of mussel (*Mytilus*

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californianus) populations *in situ* (Place, O' Donnell & Hofmann 2008; Gracey *et al*. 2008). Given the differences in scale at which these studies were performed, *c*. 2400 km and *c*. 10 m, respectively, it is necessary to understand the role of spatial scales in setting gene expression patterns. In addition, Crawford *et al*. have previously shown substantial variation in gene expression both within and between natural populations of marine fish (Oleksiak, Churchill & Crawford 2002). Furthermore, for intertidal species, microclimates created by differences in wave exposure, slope substrate and tide pools can lead to physiological differences in organisms occupying similar tide heights (see the studies by Dahlhoff & Menge 1996; Helmuth & Hofmann 2000). Such cross-scale variability raises the question of whether interindividual variation within local-scale *M. californianus* populations could overwhelm large-scale differences in gene level responses to environmental variation. If so, the use of these approaches to examine the linkages between environmental variation and species responses could be limited. In this study, we examined the variation in gene expression of *M. californianus* across three spatial scales to answer two questions:

1. Microscale (among-individual) to small-scale (within-zone) variation: Given that variation in gene expression likely exists between individuals within a single, continuous mussel bed, are patterns of gene expression largely characteristic of vertical location within the intertidal zone or does interindividual variation obscure potential between tide height differences in mussels?
2. Intermediate to regional scales (10 s of km): Are patterns of gene expression similar between sites 65 km apart, suggesting maintenance by large-scale oceanographical processes (e.g. waves, or sea water temperature), or do patterns differ between sites, suggesting acclimation or adaptation to intermediate-scale environmental variation (e.g. differences in food, or air temperature)?

To answer these questions, we profiled the gene expression patterns of mussels inhabiting locations on two separate coastal capes that display a host of ecological and physiological differences, Boiler Bay (BB) located on Cape Foulweather and Strawberry Hill (SH) located on Cape Perpetua. For instance, BB intertidal regions generally have lower mussel recruitment, slower mussel growth and weaker competition for space than SH (Menge *et al*. 1997, 2009; Menge 2004; Menge, Chan & Lubchenco 2008). Physiologically, BB mussels reproduce less, display a reduced cellular response to stress and have lower growth potential than SH mussels (Dahlhoff & Menge 1996; Halpin *et al*. 2002; Petes, Menge & Harris 2008a; Petes *et al*. 2008b). If patterns of gene expression are largely characteristic of vertical location within the intertidal zone, we would expect them to be concordant with basic physiological responses that have been established in previous studies. Thus, we can take advantage of these sites to gain insight into the applicability of these approaches in characterizing the response of mussel populations *in situ*.

Materials and methods

TIDAL HEIGHT MEASUREMENTS

Vertical elevation of each plot in the low, mid and high intertidal zones was determined using survey methods that used the local tidal datum to ensure that mussels collected experienced similar annual submersion times. The low zone was sampled just above the mean lower low water (MLLW), which corresponds to an elevation of 0 m along the eastern North Pacific coast and where organisms are submerged *c*. 90% of the time.

MUSSEL BODY TEMPERATURE ESTIMATES

To monitor relative mussel body temperatures immediately prior to sample collection, mussel-mimicking temperature data loggers were constructed by embedding *i*button data loggers (Maxim Integrated Products, Sunnyvale, CA, USA) into silicone-filled mussel shells. These mussel mimics were securely inserted into each mussel plot (see the study by Helmuth & Hofmann 2000 for details). Approximate mussel body temperature was recorded at 10-min intervals for 4 days just prior to sample collection.

CHLOROPHYLL-*A* MEASUREMENTS

Methods for quantification of abundance of phytoplankton (as proxied by chlorophyll-*a* = Chl-*a*), a primary food of mussels, followed those summarized by Menge, Chan & Lubchenco (2008). We collected bottle samples from the shore at each site by filling replicate (n = 3–5) acid-washed opaque plastic 250-mL bottles (HDP) at a depth of 30–50 cm below the surface during low tide. Fifty millilitres of water was filtered through 25-mm precombusted Whatman GF/F glass-fibre filters with a pore size of 0·7 mm. Chl-*a* concentration was determined using a Turner Designs TD-700 fluorometer after extraction in 90% HPLC acetone for 12 h in the dark at −20 °C. Prior calibrations of the fluorometer were done using pure Chl-*a* standards. Samples were taken daily to monthly, both before and during the study period (April through September).

SAMPLE COLLECTION

Mussels were sampled on successive mid-day low tides (14–15 July 2007) by cutting the attaching byssal threads with a scalpel. To investigate the effects of intra-site variation at the same and different elevations at SH, we initially performed horizontal transects at three vertical heights (referred to as SH-low, SH-mid and SH-high) within a single-wave-exposed mussel bed. We randomly collected 10 mussels from five replicate plots along the low, mid and high intertidal transects (see Fig. S1, Supporting Information). Plots were horizontally spaced at 3-m intervals. Individuals not in immediate contact with each other and with a shell length between 50 and 60 mm were otherwise randomly chosen from each plot (*c*. 0·5 m × 0·5 m). Plots with mussels inhabiting tide pools or vertical walls were excluded as these microhabitats may impact feeding habits and create a refuge from heat as the angle of incidence of incoming solar radiation is known to directly affect the organism's thermal properties (Dahlhoff & Menge 1996; Helmuth & Hofmann 2000; Denny, Miller & Harley 2006).

To evaluate inter-site variation, horizontal transects were also performed within a single wave-exposed mussel bed at BB during the next low tide series. Five replicate plots separated by 3 m were

sampled within the middle and along the upper edge of a continuous mussel bed at BB. Initially, we only intended to perform a comparison of mussels from the mid-intertidal zone from each site; however, upon surveying exact tidal height, we determined the SH-mid plots were more similar in vertical displacement to the upper edge of the mussel bed within BB (see Table S1, Supporting Information). Therefore, we sampled both the mid-intertidal zone of the mussel bed, referred to henceforth as BB-mid, and at a tidal height near the upper edge of the mussel bed more closely corresponding to tidal height of SH-mid plots, referred to as BB-high.

To test the effects of emersion stress, ten individuals from each plot were removed from the mussel bed by carefully cutting the attaching byssal threads from the substrate with a scalpel just prior to being inundated by the afternoon high tide. For five individuals, gill tissue was immediately excised and flash-frozen on dry ice. The remaining 5 individuals were placed in a 20-gallon bucket containing seawater maintained at ambient temperature (*c*. 14 °C) and O_2 saturation by repeated water changes. Mussels were allowed to recover from the emersion stress for 1 h. Following the 1-h recovery period, gill tissue was excised and flash-frozen on dry ice. All samples were maintained on dry ice and transported to UCSB where they were stored at −80 °C until used for analysis.

COMPARISON OF GENE EXPRESSION PROFILES

To determine whether variation in gene expression within a single tide height was smaller than variation across tide heights, we characterized the gene expression profile of fifteen individual samples at each tide height (three mussels per plot, five plots per tide height) from SH using a custom microarray (described below). Three of the five mussels sampled from each plot were randomly selected for transcriptome analysis to reduce the total number of arrays needed for this portion of the analysis.

For the comparison between BB and SH, RNA was pooled from the five biological replicates sampled from within each plot for BB-mid and BB-high, and reverse-transcribed for competitive hybridization and compared to the mean log ratio of SH-mid samples ($n = 5$ arrays per tidal height per treatment).

All gene expression profiles were quantified with a 4992-feature cDNA custom microarray. Microarrays were constructed from a cDNA library prepared from different tissues (gill, adductor muscle and mantle) of adult *M. californianus* mussels exposed to a variety of stressors (heat, cold, emersion, hypoxia, hypo-osmolality, cadmium and low pH) to induce a mRNA pool enriched for as many stress responsive genes as possible. For all competitive hybridizations, total RNA was extracted from the gill tissue of 30 individuals collected from a population of *M. californianus* mussels in Jalama Beach near Santa Barbara, California. These mussels were laboratory-acclimated at 14 °C for 3 weeks, but otherwise untreated, and pooled for use as a source of reference RNA. The use of nonexperimentally treated RNA reference samples is commonplace in gene expression analysis and reduces the overall number of arrays needed to perform the analyses while allowing direct comparison of expression values across samples (see the studies by Podrabsky & Somero 2004; Place, O' Donnell & Hofmann 2008). Putative gene clusters were annotated for identity using NCBI BLAST against the UniRef 90 protein data base with an evalue $\leq 10^{-4}$.

RNA was extracted from *c*. 100 mg of frozen tissue using TRIzol® (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations. Ten micrograms of total RNA was reverse-transcribed (RT) to cDNA using anchored oligo(dT15) primers and amino-allyl dUTP, fluorescently labelled and hybridized to a cDNA microarray as previously described by Place, O'Donnell & Hofmann (2008). Microarrays were printed and a 9-mer Cy3-labelled spot QC and red reflection spot QC were performed at the Oregon State University Center for Genome Research and Biocomputing. All microarrays were stored in a light-protected desiccation cabinet until use. The microarrays were scanned on an AXON GenePix 4000B microarray scanner (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA). Spot intensity data were extracted using GENEPIX PRO 4.0 software, and the ratio of Cy5 to Cy3 fluorescence was quantified for each spot on the arrays (Axon Instruments).

The data discussed in this publication along with microarray printing and QC procedures have been deposited in NCBIs Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE21088.

NORMALIZATION AND STATISTICAL ANALYSIS OF MICROARRAY DATA

For each array, spatial and intensity-based trends in the data were removed by Lowess normalization of fluorescence data using GENESPRING GX 10.0 software (Agilent, Santa Clara, CA, USA). This removes data artefacts introduced by slightly different biochemical properties associated with the dyes (see the study by Draghici 2003). Features that were not represented by a minimum spot intensity in at least 75% of the samples were excluded from further consideration. In addition, only those features that displayed a signal-to-noise ratio (SNR) of ≥3 were included in further analysis.

Principal component analysis (PCA) was performed on the combined mean $\log_2$ ratio (635/532) for biological replicates sampled within a single plot using GENESPRING GX 10.0. This allowed us to determine the cumulative separation between sites, vertical position on the shore, and recovery, as a function of numerous gene level responses ($n = 5$ arrays/tidal height/treatment). The results of the PCA were projected as a loading plot to illustrate the degree of similarity between transcriptomes.

To assess the impacts of tidal height on gene expression during and after an emersion stress within a site, we used nonparametric significance tests (Mann–Whitney–Wilcoxon) to compare the median $\log_2$ ratio (635/532) of paired features from individual mussels taken from SH-low, SH-mid and SH-high plots, and identified features whose expression displayed significant changes in expression in one or more of the tide heights. All *P*-values were adjusted with the Benjamini and Hochberg false discovery rate (FDR) correction for multiple testing (FDR = 0·25) and were considered significant if $P < 0{\cdot}05$.

As we were interested in examining the interaction of geographical location and vertical position on the shore, we next performed a two-way ANOVA with collection site and tide height as fixed variables. As before, *P*-values were adjusted with the Benjamini and Hochberg method (FDR = 0·25). We then performed *k*-means clustering analysis on the $\log_2$ ratio data for all genes displaying a significant change in gene expression for tide height or 1-h recovery (FDR-corrected $P < 0{\cdot}05$). *k*-Means clustering was performed using the Pearson's uncentred distance metric within the GENESPRING GX 10.0 gene expression analysis software package.

Biological function of the gene sets described by the clusters identified through the *k*-means analyses were further characterized by over-representation analysis (ORA) in ErmineJ software package (Lee *et al*. 2005). Parameters for the ORA were as follows: minimum gene

set size = 5, maximum gene set size = 200, FDR correction Benjamini-Hochberg method. FDR-corrected *P*-values < 0·1 were considered significant in accordance with recommended practices (Lee *et al.* 2005).

Results

TIDAL HEIGHT SURVEYS

The mean tidal heights at which mussels were sampled from within BB and SH mussel beds as determined by surveys of elevation were as follows: BB-mid (1·44 ± 0·59 m), BB-high (1·68 ± 0·69 m), SH-low (1·00 ± 0·42 m), SH-mid (1·75 ± 0·72 m) and SH-high (2·07 ± 0·85 m) above MLLW. Elevations for individual plots are reported in the Table S1 (Supporting information).

BODY TEMPERATURE ESTIMATES

Due to loss of mussel data loggers after the initial deployment at BB and SH, we are unable to provide comparisons for the entire week prior to collection. However, we were able to collect detailed overlapping temperature estimates for mussel body temperature for 5 days during the experiment. For the 5 days in which we can directly compared body temperature estimates, BB-mid mussels displayed elevated daily mean body temperature estimates, 15·52–17·09 °C, compared with SH-mid, 13·33–15·10 °C (Fig. 1a). BB-mid mussels also show greater temperature variance during this period (Fig. 1a). Daily minimum temperatures (set by sea surface temperatures) were similar at both sites, ranging between 11 and 14 °C. But daily maxima were higher at BB-mid, with daily maxima routinely recorded above 20 °C, while daily maximum temperatures recorded for SH-mid rarely reached above 20 °C (Fig. 1a). Alternatively, mussels inhabiting the high intertidal zone displayed more similar daily maximal body temperatures despite differences in vertical location on the shore (Fig. 1b). Seasonally, mussel body temperatures in the mid-intertidal zone of BB and SH are similar with respect to maximal daily temperatures, suggesting that differences in physiological responses to temperature changes would be unlikely to result from simple differences in thermal acclimatization for mussels located along these two capes (Fig. 1c). Daily mean, maximum and minimum temperatures for the mid- intertidal and high intertidal zones at each site are reported in the supplementary material (see Table S2, Supporting Information).

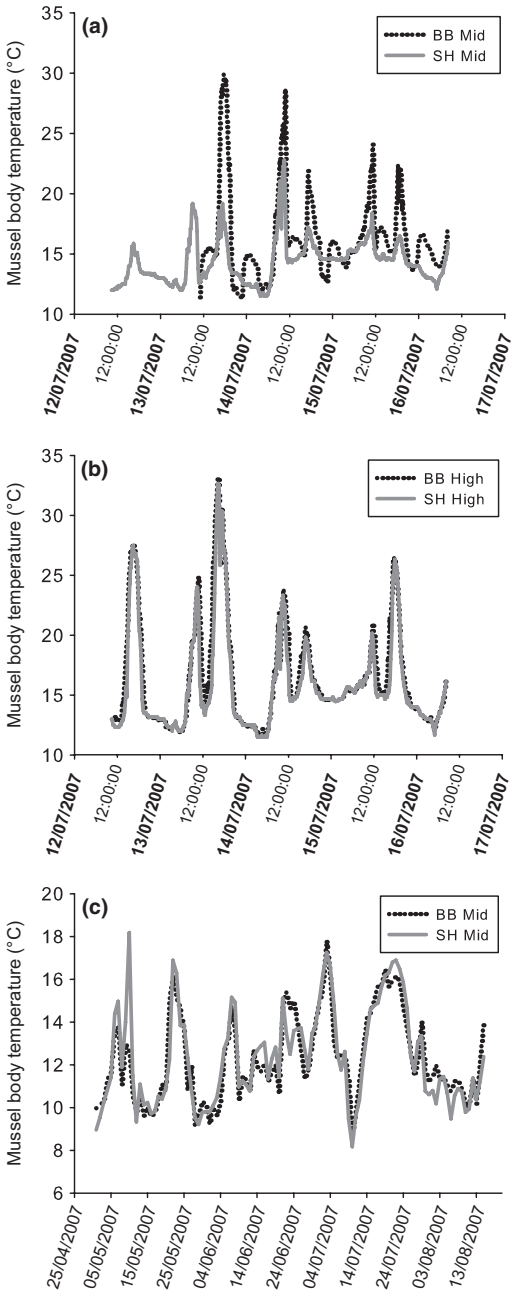

Fig. 1. Temperature traces of the estimated body temperature experienced by mussels located in the mid-intertidal zone (a) or high intertidal zone (b) at Boiler Bay (Black dotted line) and Strawberry Hill (grey solid line) for the week during the low tide series during which mussels were sampled. (c) Estimated daily maximal body temperature experienced by mussels located in the mid-intertidal both before and during the study period (April through August).

CHLOROPHYLL-*A* MEASUREMENTS

Bottle samples taken from the shore at each site show markedly higher levels of Chl-*a* in the upper water column at SH when compared with BB (Fig. 2). From May through August, Chl-*a* concentrations at SH are at least two fold higher than at BB, with an increase to nearly 20-fold in mid June (Fig. 2). Chl-*a* levels were at least 10-fold higher at SH for the month preceding our collection (Fig. 2).

INTER-SITE COMPARISONS OF VARIATION IN GENE EXPRESSION

To gain a measure of the global variation in gene expression among mussels that are (i) spatially separated within a single mussel bed and (ii) experiencing and recovering from mid-day aerial exposure, we performed a PCA of the gene level responses in mussels from a single mussel bed with or without a 1-h recovery period in ambient seawater (Fig. 3).

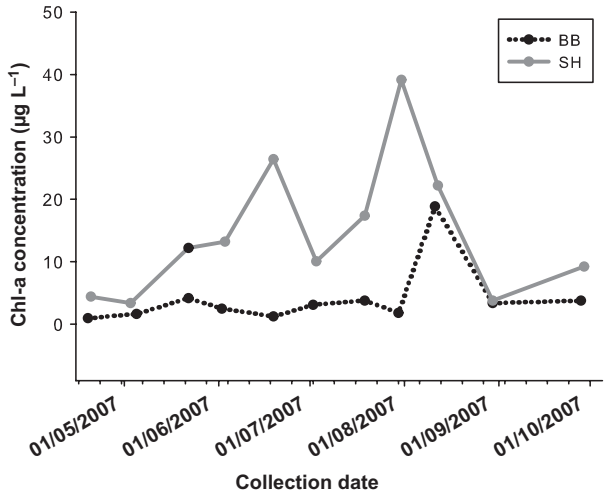

Fig. 2. Chlorophyll-*a* concentrations taken via bottle sampling performed at Boiler Bay, OR (Black dotted line) and Strawberry Hill, OR (grey solid line). Samples were taken daily to monthly, both before and during the study period (April through September).

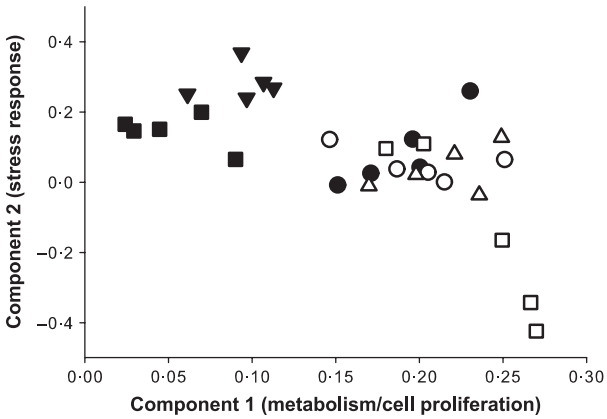

Fig. 3. Principal component analysis of the cumulative separation in gene expression between mussels within a single mussel bed located at Strawberry Hill, OR. Mussels were sampled from three separate vertical positions on the shore (low – circles, mid – triangles and high – squares). Mussels were sampled during daily emersion (closed symbols) or after a 1-h recovery in ambient seawater (open symbols). Plot ordination was performed for the first two component loadings – Component 1: *x*-axis (80·01% of the variation); Component 2: *y*-axis (12·23% of the variation).

As expected, mussels inhabiting different vertical heights along the shore show strong variation in their gene level responses during both aerial exposure and after a 1-h recovery, specifically with respect to the first principal component (PC1). Annotation of the top 100 features whose expression patterns most contributed to PC1 identified several genes involved in metabolic processes (Fig. 3, see Table S3, Supporting Information). In contrast, no significant variation in gene expression patterns was detected among mussels inhabiting the same level on the shore during an emersion event even if separated horizontally by as much as 15 m (Fig. 3). Only SH-high plots given a 1-h recovery displayed any variation in their gene level responses with respect to horizontal separation along the shore (Fig. 3, open squares). No discrete shifts in gene expression were detected between SH-low mussels given a 1-h recovery in ambient seawater and those sampled immediately after an emersion event (Fig. 3, circles). During a mid-day emersion event, SH-mid and SH-high mussels displayed similar gene expression patterns that deviated from SH-low mussels with respect to PC1 (Fig. 3). After a 1-h recovery period, gene expression patterns of SH-mid mussels and two of the five plots for SH-high mussels were no longer distinguishable from those displayed by SH-low mussels (Fig. 3). Furthermore, for three of the five SH-high plots sampled, a shift in gene expression patterns with respect to both PC1 and PC2 had occurred after the 1-h recovery, resulting in a distinct clustering of these mussels (Fig. 3, open squares).

To identify genes that showed significant changes in gene expression, we used nonparametric significance tests to compare the median $\log_2$ ratio (635/532) of paired features from individual mussels taken from SH-low, SH-mid and SH-high plots. Volcano plots were constructed by plotting the negative $\log_{10}$ of the *P*-value against the $\log_2$ of the fold change between the two conditions under consideration (low/mid, low/high or mid/high). Highlighted data points represent the genes meeting the applied cut-offs (fold change ≥2, FDR-corrected $P < 0{\cdot}05$) used to identify both biologically (magnitude) and statistically significant changes for each comparison (Fig. 4a–f).

Of the nearly 400 genes that showed differences in gene expression between tide heights (MWW, FDR-corrected $P < 0{\cdot}05$), 68 genes from the mid-intertidal population and 167 genes from the high intertidal population displayed at least a two fold change in expression relative to the median $\log_2$ ratio (635/532) of low intertidal populations within the SH mussel bed (Fig. 4a,c respectively). Classification of these genes by their biological function listed in the Gene Ontology (GO) data base verifies the trends in the PCA (Fig. 3), with several genes associated with metabolic processes [*aldehyde dehydrogenase* (*ALDH*), *acetyl-coenzyme A synthetase* (*ACSA*), *low-density lipoprotein receptor-related protein1* (*LRP1*), *ubiquinol-cytochrome C reductase* (*Uqcrfs1*)] being down-regulated in both SH-mid and SH-high mussels (see Table S4, Supporting Information). Subsequent to a 1-h recovery period in ambient seawater, the number of different genes between the SH-mid and SH-low mussels dropped to only 19 (Fig. 4b). In contrast, the difference between SH-high and SH-low mussels widened, with the number of differentially expressed genes increasing to 336, with a strong up-regulation of multiple stress response genes. Included among these genes were *heat-shock cognate 71*(*Hsc71*), *heat-shock protein 90-alpha* (*Hsp90-α*), *metallothionein 20-III isoform B* (*MT-20 IIIb*), *ubiquitin* (*Ub*) and *stress-induced-phosphoprotein-1* (*STI3*) (Fig. 4d, Table S4, Supporting Information). A similar pattern was seen with respect to SH-mid compared with SH-high intertidal mussels, with the number of genes increasing from 28 during an emersion stress to 103 genes identified after a 1-h recovery (Fig. 4e, f respectively).

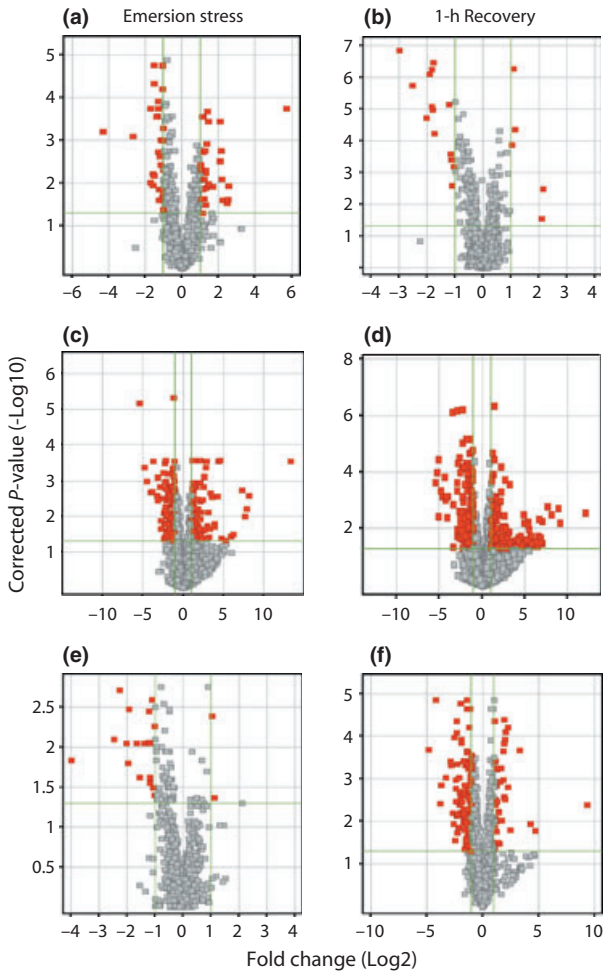

Fig. 4. Volcano plots showing the pairwise comparison of the differential expression of genes between mussels at three separate vertical positions on the shore during an emersion stress or after a 1-h recovery in ambient seawater. The red points indicate genes-of-interest that display both large-magnitude fold changes (FC ≥ 2, *x*-axis) as well as high statistical significance ($P < 0{\cdot}05$, *y*-axis). Emersion stress: (a) Strawberry Hill (SH)-low vs. SH-mid, (c) SH-low vs. SH-high, (e) SH-mid vs. SH-high. 1 h recovery: (b) SH-low vs. SH-mid, (d) SH-low vs. SH-high, (f) SH-mid vs. SH-high.

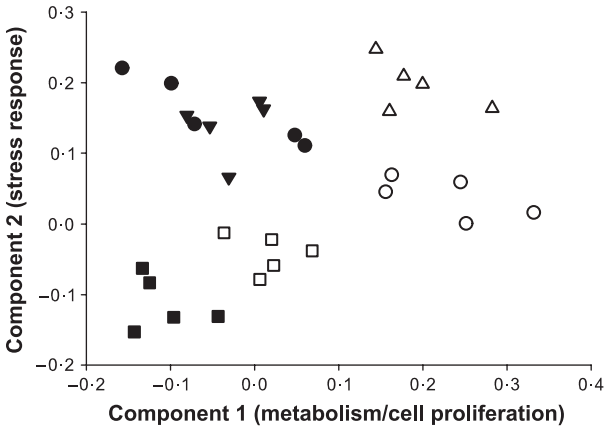

Fig. 5. Principal component analysis of the cumulative separation in gene expression between mussel populations located at Strawberry Hill (SH) and Boiler Bay (BB), OR. Mussels were sampled from the mid-intertidal zone at SH (squares) and two separate vertical positions on the shore at BB (mid – triangles and high – circles). Mussels were sampled during daily emersion (closed symbols) or after a 1-h recovery in ambient seawater (open symbols). Plot ordination was performed for the first 2 component loadings - Component 1: *x*-axis (35·13% of the variation); Component 2: *y*-axis (30·87% of the variation).

INTRA-SITE COMPARISONS OF VARIATION IN GENE EXPRESSION

To understand the extent of separation in physiological responses of mussels inhabiting two biogeographically distinct capes, we used PCA to gauge the cumulative variation of the gene level responses. PCA was performed on the gene expression patterns of mussels sampled at five different locations along horizontal transects, either immediately prior to inundation by the rising tide or 1 h after re-immersion in ambient seawater. The clustering of individuals from within a site is indicative of distinctive gene expression patterns between sites (Fig. 5). Similar to the pattern of gene expression illustrated in the previous PCA plot (Fig. 3), SH-mid mussels showed a distinctive shift in gene expression when recovering from emersion with respect to PC1 (Fig. 5). Similarly, mussels sampled from plots located within the mid-intertidal and high intertidal zones for BB showed a strong shift with respect to PC1 (Fig. 5). Reinforcing what we observed within the mussel population at SH, mussels sampled from BB-mid and BB-high intertidal plots showed strong similarity in their gene expression signatures during the emersion event (Fig. 5). However, within 1 h of recovery at ambient seawater temperatures, the signatures of BB-mid and BB-high mussels were clearly distinguished from one another with respect to PC2 (Fig. 5). Investigation of the top 100 features contributing to PC2 revealed a number of genes with important roles in stress response (see Table S5, Supporting Information). Although SH-mid-intertidal mussels were collected from similar elevations as the BB-high plots, and the same relative location within the mussel bed as the BB-mid plots, the SH-mid mussels showed distinct differences in their gene expression patterns based largely on the magnitude of the gene expression event. In fact, the gene expression patterns of mussels located within BB-high plots were more closely aligned to BB-mid plots despite occupying the same vertical position on the shore as mussels taken from SH-mid plots (Fig. 5).

Pairwise comparison of BB-mid to SH-mid plots identified 102 differentially expressed features during the emersion event and 188 differentially expressed features after a 1-h recovery in ambient seawater (see Fig. S2 and Table S6, Supporting Information). Consistent with the lower body temperatures recorded in SH-mid mussels (Fig. 1a), examination of the biological processes associated with the genes differentially expressed after a 1-h recovery revealed a general down-regulation of stress response genes, such as *Hsc71*, *78-kDa glucose-regulated protein* (*Grp78*) and *Ub*, in SH-mid mussels (Table S6, Supporting Information). In addition, SH-mid

mussels displayed an up-regulation of metabolic genes, such as *ALDH* and *LRP1,* compared with BB-mid mussels (Table S6, Supporting Information), consistent with the increased food availability at SH (Fig. 2).

GENE INTERACTIONS AND FUNCTIONAL CLASSIFICATION

In all, 648 features displayed significant changes in gene expression in response to at least one of the two parameters tested (Collection site & Tide-height). The relationships and interaction terms of genes identified within each condition are illustrated in Fig. S3 (Supporting Information). Of 648 features identified, most features (451) varied with geographical location, 167 features varied with vertical location within the two mussel beds and variation of only 30 features was context dependent (interaction terms were significant; Fig. S3, Supporting Information). Of the 30 features displaying an interaction, two-thirds were found to overlap with the gene list for collection site (7), tidal height (5) or both (8) (Fig. S3, Supporting Information).

k-means clustering of the 648 features identified in the variance analysis described earlier produced eight gene set clusters that displayed similar characteristics of gene expression (data not shown). Functional characterization of these eight gene sets by ORA revealed key functional categories that were enriched in only two of the eight clusters. Six of the clusters were functionally unclassifiable, potentially because of the large number of unknown features. Cluster A was enriched for protein folding (GO:0006457, FDR-corrected $P = 0{\cdot}0463$), while cluster C was enriched for metabolic processes (GO:0008152, FDR-corrected $P = 0{\cdot}0814$). Annotated heat maps of the genes for which function could be assigned highlight the clustering and normalized expression of gene sets with enrichment for GO biological process classification (Fig. 6). The genes that show the largest difference in response to emersion stress are members of the molecular chaperone family *Hsc71, Hsp70* and *Hsp90-α* (Fig. 6a). Even though mussel body temperatures from the high intertidal plots were similar between BB and SH during the mid-day exposure (Fig. 1b; Table S1, Supporting Information), we saw an exaggerated response in genes associated with cellular stress in cluster A (Fig. 6a). Consistent with previous trends, expressions of genes associated with metabolism appear suppressed during emersion, especially in the high intertidal plots where the down-regulation of these genes appeared to continue into the first hour of re-immersion in ambient seawater (Fig. 6b). Lastly, the level of expression for genes associated with cell cycle control, G2/mitotic-specific cyclin B, cell division cycle 2-related protein kinase and cell division protein ftsH homolog, remains low even in mid-intertidal mussels in which up-regulation of metabolic genes has resumed (Fig. 6b).

Discussion

In our analysis of gene-level responses of *M. californianus*, we identified two trends in biological function that offer insight into the mechanisms linking the environment to organismal response at the population level. First, significant variation of the transcriptome can be attributed to changes in genes involved in primary metabolic pathways. For mussels inhabiting different elevations within the rocky intertidal, both within a single continuous mussel bed and within populations that span biogeographically distinct capes, metabolic processes were associated with the principal component that accounts for the greatest fraction of variation in gene expression. Temporally, mussels inhabiting two different capes separated by *c.* 65 km display remarkably similar metabolic responses despite occupying different tide heights on shore. Differences between them were primarily in the magnitude of responses, rather than in the pattern of responses. These data suggest that oceanographical level processes can link physiological responses across intermediate scales and may highlight the important role that metabolic state may play in the response of these populations. Second, expression of genes involved in the highly conserved cellular stress response (CSR), a coordinated set of genomic responses that protect the cell against environmental stress (Kültz 2003, 2005), consistently differentiated mussels across both small and intermediate spatial scales and may have secondary impacts on the oscillation of the expression of genes associated with metabolic pathways observed in these populations.

Our results suggest that within a mussel bed, vertical position on the shore may be the dominant determinant of gene expression patterns and potentially physiological responses. This supports our hypothesis that gene expression patterns are more characteristic of absolute rather than effective tide height. Although microhabitat impacts individual physiological traits and can result in 'effective tide height', or the stimulation of responses that are associated with absolute elevation (Okamura 1992; Williams & Somero 1996; Helmuth & Hofmann 2000; Halpin *et al.* 2002), our data suggest that vertical height on the shore provides a strong metric for predicting local organismal responses and limits to changing climates.

When the latitudinal separation is increased from metres to tens of kilometres, our data suggest the dominant drivers of variation at the level of the transcript shifts from vertical tide height to oceanographical processes varying over intermediate spatial scales. Our expression profiles identified over 600 features that differed in the relative level of expression when collection site or tide height was considered. Collection site alone accounted for most of the features showing at least a two fold change in expression. In addition, analysis of the expression profiles of mussels sampled from plots at both SH and BB shows stronger similarities in expression signatures for mussels taken within the same site, even when sampled from different tide heights. Hence, strong physical linkages formed between mussel populations and intermediate-scale oceanographical processes may overwhelm local-scale differences in tidal elevation. These findings only partially support our second hypothesis that gene expression patterns are set by large-scale oceanographical processes, as it appears that the mechanism by which these processes act are themselves dependent on scale.

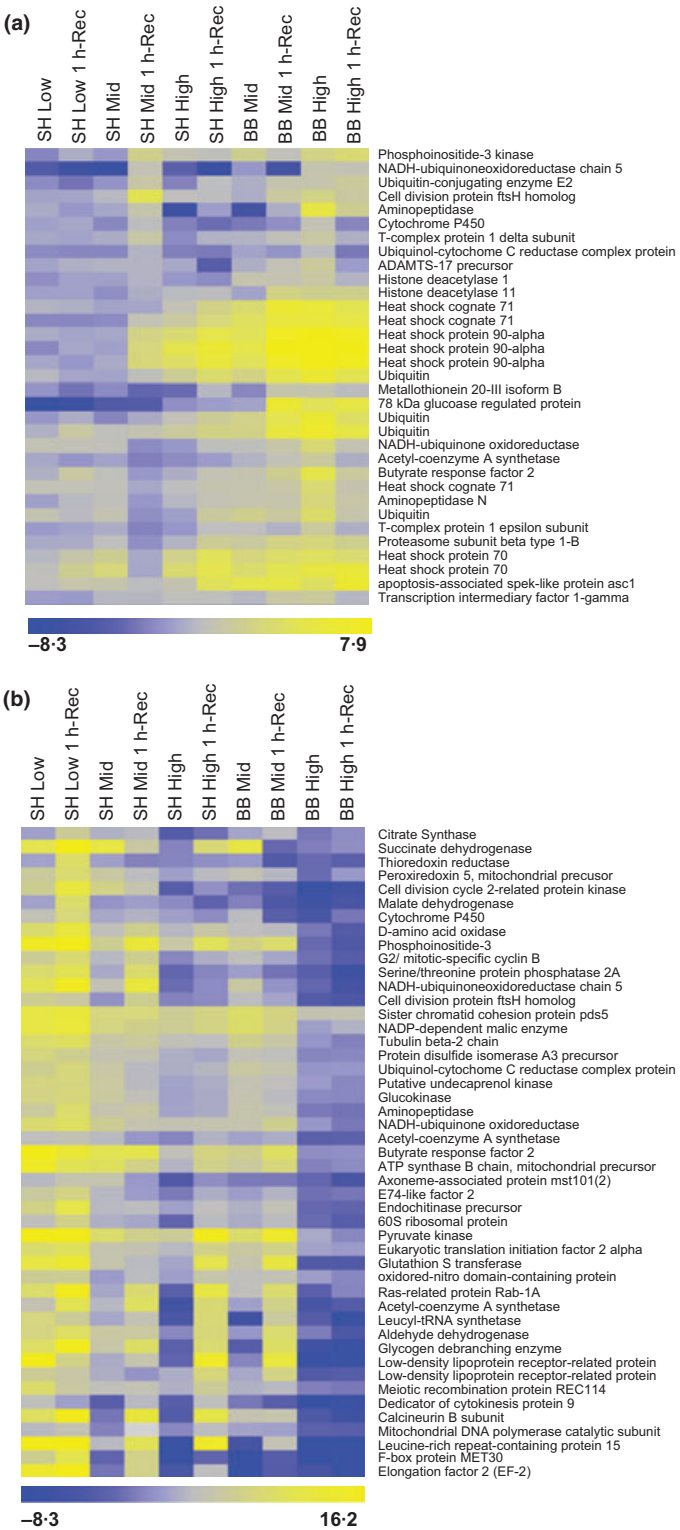

Fig. 6. Annotated heat maps showing the relative expression [down-regulated (blue); up-regulated (yellow)] of gene sets identified in the *k*-means clustering analysis with over-representation of genes linked to a specific Gene Ontology (GO) biological classification. Over-representation analysis of the k-means clustering results identified to key functional categories that were significantly over-represented: (a) protein folding [GO:0006457, false discovery rate (FDR)-corrected $P = 0{\cdot}0463$] and (b) metabolic processes (GO:0008152, FDR-corrected $P = 0{\cdot}0814$).

FOOD AVAILABILITY AND METABOLISM

Historically, phytoplankton abundance has been lower at BB compared with SH (Menge *et al*. 1997, 2009), a trend that held during our study. In addition to the underlying oceanographical differences resulting in lower food availability at BB, local variation across smaller spatial scales can also

impact availability and utilization of resources. Among other factors, vertical location within the intertidal zone significantly impacts the window of time sessile organisms have to feed and thus may be a strong environmental driver of the physiological response of mussels within a population. From an ecological perspective, food availability at a given geographical location, in combination with the feeding window, has long been implicated in differences in growth rates and reproductive output (Wingfield & Sapolsky 2003; Petes, Menge & Harris 2008a; Petes *et al*. 2008b). Feeding state also influences circadian rhythms (Sonoda *et al*. 2007) and thus has been proposed as a mechanism that may act to gate the metabolic response of an intertidal organism subjected to daily tidal variation (Gracey *et al*. 2008). Therefore, if the magnitude and frequency of transcriptional variance are indeed linked to environmental parameters, we would expect the variation in metabolic responses to be reflected in food availability, and in the feeding window of the mussels as a function of elevation within the intertidal zone. Indeed, when we investigate the metabolic response of mussels inhabiting different elevations as a proxy for feeding window, we find qualitative responses of metabolic genes that are largely reflective of this scenario.

At the level of the transcript, low intertidal mussels altered their physiology very little with respect to the tide cycle. Thus, although these mussels were emersed during daylight spring tides, the frequency and duration of exposure evidently were not sufficient to result in meaningful variation in gene expression patterns. Although we only tracked gene expression changes that occurred with the first hour of re-submergence and cannot rule out the presence of longer-term variation, these data are consistent with the lack of transcriptional changes reported for *M. californianus* inhabiting the low intertidal zone of Monterey Bay over the course of a 3-day tide series (Gracey *et al*. 2008).

For mussels inhabiting the mid-intertidal and high intertidal zones, a reduction in the expression of a cluster of genes involved in metabolic processes such as *pyruvate kinase*, *ATP synthase β chain*, *LDL receptor-related protein 1* and *acetyl-coA synthetase* suggests that their metabolic capacity was suppressed during emersion. For SH-mid mussels, the relative expression of this gene cluster reached levels comparable to those seen in low intertidal mussels within an hour of re-immersion. Although the links between gene expression changes and functional responses need to be further verified in this system, these gene expression patterns suggest that SH-mid mussels quickly resume normal respiration despite reducing metabolic capacity during an emersion event.

As seen with the initial intra-site analysis, inspection of the top 100 features from PC1 of the inter-site PCA revealed several genes whose biological function was associated with metabolic activities. Unlike the gene expression signatures seen in SH, mussels sampled from BB-mid and BB-high plots yielded similar metabolic responses both prior to and 1h after re-immersion. Furthermore, when the gene-level responses of mussels from BB are compared with mussels sampled from the same tidal height in SH, the expression patterns display similar qualitative responses, but the magnitude of the response was exaggerated in mussels sampled from BB. This variation may be evidence of direct links between metabolic state and physiological perception of environmental differences in food availability at intermediate scales.

Dahlhoff & Menge (1996) suggested that the effects of stress might be amplified in organisms if the occurrence of abiotic stress coincides with times of low productivity. Two more recent studies provide further empirical evidence of this interaction. In bivalves, hypoxia alone was found to have a weak effect on RNA, scope for growth and morphometric indices (Norkko *et al*. 2005). However, when food availability was reduced, the impact of hypoxia was amplified (Norkko *et al*. 2005). Similar interactions were reported in a study assessing the effects of food availability and elevated body temperature on the survival of two *Mytilus* congeners (Schneider, Van Thiel & Helmuth 2010). In this study, Schneider, Van Thiel & Helmuth (2010) reported a 24% difference in survival at 30 °C between mussels given a low or high food treatment. These findings are similar to ours for the potential role of food availability in setting gene expression patterns at different scales. Our data suggest the variation in gene expression of metabolic genes is likely driven in large part by phytoplankton abundance at intermediate scales, in addition to feeding windows at different tidal heights within a site. Taken together, these data highlight the different mechanisms by which environmental parameters can act, and continue to underscore the need to understand physiological responses across multiple scales.

TEMPERATURE AND THERMAL WINDOWS OF PERFORMANCE

In their consideration of thermal tolerance windows, Frederich & Pörtner (2000) discuss three distinct physiological transitions that may define the progression of temperature impacts in ectothermic organisms. The first transition that occurs has been termed the 'pejus' temperature (T_p) and is marked by the onset of oxygen limitations in aerobic scope, which is followed by passive tolerance tactics induced by a critical temperature (T_c). The last transition, brought on by exposure to denaturing temperatures (T_d), is characterized by loss of structural integrity of macromolecules and the induction of protective mechanisms such as the CSR (Frederich & Pörtner 2000). The relative small number of gene expression changes observed in SH-low mussels during an extreme low tide would suggests that body temperatures rarely exceed the thermal windows for optimal growth and performance in these mussels. In contrast, during low-tide emersion, gene expression patterns observed in mussels sampled from the mid- intertidal and high intertidal plots may be an early indicator that the T_p threshold has been reached. Lastly, during the first hour of re-immersion, we see induction of pathways associated with protein rescue, cellular repair and protein degradation, in addition to genes involved in oxidative stress pathways in a majority of SH-high mussels. This may be an indication that a large number of SH-high mussels may cross

the T_d threshold during low-tide emersion and incur significant energetic debts because of macromolecular damage.

Gene expression patterns also appear to be strongly influenced by temperature across intermediate scales, but mussels sampled from BB did not show the strong influence of zonation on thermal windows as was seen at SH. Unlike SH-mid mussels that display gene expression patterns that would suggest they remain below the T_d threshold, both BB-mid and BB-high mussels display gene expression patterns that may indicate that they have surpassed the T_d threshold. This, despite the fact that they were existing at vertical elevations equivalent to, and in some cases lower than, mussels within the mid-intertidal zone of SH. The increased solar heating seen in BB-mid mussels is likely the primary factor underlying the clear distinction between the gene expression signatures of mussels sampled from SH-mid and BB-mid plots with respect to stress response genes. However, the expression patterns seen in BB-high mussels are not as easily explained. Despite experiencing similar body temperatures during the mid-day exposure, BB-high mussels exhibit a more pronounced stress response compared with SH-high mussels. These data suggest the capacity for maintaining cellular homeostasis may be reduced in BB populations and that growth and fitness could be compromised for extended periods even at moderate elevations.

In addition to thermal insults, reactive oxygen species (ROS) also appear to be important modulators of cellular responses during the first hour of recovery from aerial exposure. A host of organisms show metabolic depression during cellular stress events, and it has been postulated by some that this metabolic depression is related to an attempt to reduce the accumulation of ROS (see Lesser 2006 for review). Production of ROS has been implicated in the transcriptional regulation of *CYP* isoforms and may serve as negative feedback regulators of *cytochrome P450* (Barouki & Morel 2001). During aerial emersion, we observed down-regulation of genes involved in aerobic respiration that was accompanied by a nearly 15-fold lower expression level of *cytochrome P450* mRNA in SH-high mussels relative to SH-low mussels. Furthermore, within an hour of re-immersion, SH-high mussels showed up-regulation of the antioxidant genes, *Perodoxin 5* and *glutathione S-transferase*. These data are consistent with reported high levels of carotenoids, believed to play an important role in mitigating oxidative stress in high intertidal mussels (Petes, Menge & Harris 2008a; Petes *et al.* 2008b). More recently, transcriptomic analysis of the thermal stress response of laboratory-acclimated *Mytilus* congeners has described similar patterns of simultaneous down-regulation of metabolic genes and up-regulation of oxidative stress genes (Lockwood, Sanders & Somero 2010). Parallel proteomic analysis of these mussels verifies similar functional changes at the level of the protein, supporting the link between changes at the level of transcript and physiological changes at the level of the organism (Tomanek & Zuzow 2010). Taken together, these data provide further support that SH-high mussels demonstrate cellular responses consistent with an organism that has crossed the T_d threshold.

Conclusions

The complex linkages between physiological responses and environmental variation present a major challenge to understanding the consequences of global climate change for species. In this study, we have provided an initial glimpse into some of these complex interactions, raising the possibility that the mechanisms by which food availability and temperature work to set gene expression patterns can differ at small and intermediate scales. In addition, our results highlight potentially compounding effects of temperature and food availability, pointing to a mechanism by which biotic and abiotic variables may work together to lower physiological limits and set geographical boundaries. For instance, the induction of stress response pathways may come at a substantial energetic cost to other biological functions, and that gene expression may result in a trade-off between increased fitness and growth rate (e.g. Lang, Murray & Botstein 2009). If this energetic cost is accrued within a low or poor nutrient environment, an organism may lack sufficient energetic reserves to mount a proper cellular defence, resulting in a magnification of the insult. As a result of these compounding effects, mussel populations inhabiting waters characterized by low productivity may be living closer to their thermal physiological limits than previously estimated. These results could have important implications for future efforts aimed at predicting the impact of climate change on the connectivity of populations across large biogeographical scales where distinct oceanographical features work to constrain range limits through physiological tolerance.

Acknowledgements

We thank all the members of the Hofmann and Menge Lab groups for assistance with sample collection, especially Dr. Michael O'Donnell for his contributions to field collections. Dr. Francis Chan oversaw collection of the phytoplankton samples and provided the data for Fig. 2. The authors acknowledge the U.S. National Science Foundation for financial support during the course of this writing project (NSF grants OCE-0425107 to G.E.H. and OCE-0726983 to B.A.M.). S.P.P. was supported by a U.S. National Institutes of Health NIH-NRSA Postdoctoral Fellowship during a portion of this project. B.A.M. was also supported by grants from the D. and L. Packard Foundation, the G. and B. Moore Foundation, the A. W. Mellon Foundation, and the Valley Foundation. This is contribution number 403 from PISCO, the Partnership for Interdisciplinary Studies of Coastal Oceans funded primarily by the Gordon and Betty Moore Foundation and David and Lucile Packard Foundation.

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Received 29 March 2011; accepted 9 September 2011
Handling Editor: Sue Jackson

Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1. Surveyed elevation of sampling plots.

Table S2. Daily mussel body temperature estimates.

Table S3. Gene list of annotated genes from features in PCA depicted in Figure 3.

Table S4. Annotated Genes of interest produced by pair wise comparisons depicted in Figure 4.

Table S5. Gene list of annotated genes from features in PCA depicted in Figure 5.

Table S6. Annotated Genes of interest produced by pair wise comparisons depicted in Figure S2.

Figure S1. Map of sampling sites along the Oregon coast depicting the two different spatial scales at which gene expression was assessed.

Figure S2. Volcano plots showing the pair-wise comparison of the differential expression of genes between mussels sampled at the mid-intertidal zone at BB and SH.

Figure S3. Venn diagram showing the relationship of differentially expressed genes identified through pair-wise comparison of the gene expression between mussel populations at Boiler Bay and Strawberry Hill.

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