H−1 NMR Metabolomics Reveals Contrasting Response by Male and Female Mussels Exposed to Reduced Seawater pH, Increased Temperature, and a Pathogen

ARTICLE *in* ENVIRONMENTAL SCIENCE AND TECHNOLOGY · MAY 2014

Impact Factor: 5.33 · DOI: 10.1021/es501601w · Source: PubMed

Environmental Science & Technology

Article

pubs.acs.org/est

^{1}H NMR Metabolomics Reveals Contrasting Response by Male and Female Mussels Exposed to Reduced Seawater pH, Increased Temperature, and a Pathogen

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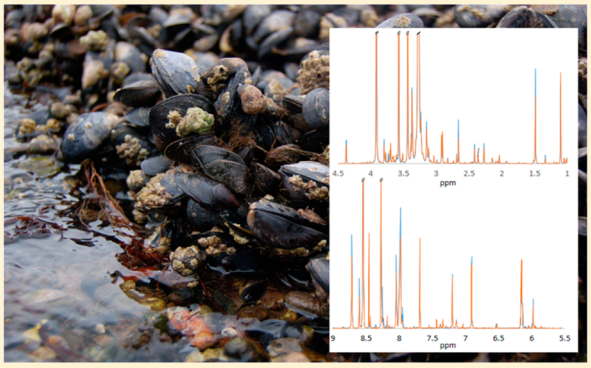
Supporting Information

ABSTRACT: Human activities are fundamentally altering the chemistry of the world's oceans. Ocean acidification (OA) is occurring against a background of warming and an increasing occurrence of disease outbreaks, posing a significant threat to marine organisms, communities, and ecosystems. In the current study, ^{1}H NMR spectroscopy was used to investigate the response of the blue mussel, *Mytilus edulis*, to a 90-day exposure to reduced seawater pH and increased temperature, followed by a subsequent pathogenic challenge. Analysis of the metabolome revealed significant differences between male and female organisms. Furthermore, males and females are shown to respond differently to environmental stress. While males were significantly affected by reduced seawater pH, increased temperature, and a bacterial challenge, it was only a reduction in seawater pH that impacted females. Despite impacting males and females differently, stressors seem to act via a generalized stress response impacting both energy metabolism and osmotic balance in both sexes. This study therefore has important implications for the interpretation of metabolomic data in mussels, as well as the impact of environmental stress in marine invertebrates in general.



INTRODUCTION

Anthropogenic climate change poses a significant threat to marine ecosystems.[1] Atmospheric concentrations of CO_2 recently exceeded 400 ppm for the first time since records began,[2] with increasing CO_2 concentrations already having led to measurable changes in ocean carbonate chemistry and seawater temperature.[3] With CO_2 emissions predicted to continue unabated, surface seawater pH is projected to drop a further 0.4 units and sea surface temperatures increase by between 1 and 4 °C by the end of the current century.[3,4] Moreover, in a heterogeneous and naturally variable world, environmental stressors seldom occur in isolation.[5] Ocean acidification (OA) is occurring against a background of pollution[6] and disease outbreaks,[7] posing a significant challenge to the many marine organisms that inhabit coastal regions. It is therefore vital to consider the impact of these environmental costressors concurrently, over a broad exposure range.[4]

Metabolomics has great potential to increase our understanding of anthropogenic climate change impacts in marine ecosystems.[8] By characterizing a suite of low molecular weight endogenous metabolites within a biological sample,[9,10] this technique provides information on the functional status of an organism, related directly to its phenotype.[11,12] It therefore holds great promise as a research tool for environmental risk assessment[8] by elucidating an organism's ability to respond and/or adapt to changing conditions.[13] However, this field is still in its infancy with respect to its application to OA, with only two published studies to date. Lannig et al. investigated the impact of OA and acute temperature shock on the Pacific oyster, *Crassostrea gigas*.[14] They reported a significant OA-related shift in the oyster's metabolic pathways. Hammer et al.[15] found that exposure to elevated CO_2 resulted in a significant reduction in the levels of important intracellular osmolytes in the crab *Carcinus maenas*, pointing to OA-related disruption of osmotic regulation. While providing a novel mechanistic understanding of CO_2 impacts in two marine invertebrate species, neither study considered that there may be sex-related

Received: April 1, 2014
Revised: May 20, 2014
Accepted: May 20, 2014

   dx.doi.org/10.1021/es501601w | Environ. Sci. Technol. XXXX, XXX, XXX−XXX

differences. Phenotypic anchoring, or the interpretation of metabolic data in the context of life history information (e.g., age and sex), is a key principle in environmental metabolomics.[16] Sex may significantly influence an organism's metabolic processes, as illustrated by metabolic variability in mussels exposed to environmental hypoxia.[17] Not considering sex may mask more subtle, sex-specific responses to environmental stress. Consequently, to interpret the metabolic fingerprint of an impacted organism, and accurately identify any biomarkers in relation to exposure scenarios, it is crucial to fully understand and account for the phenotype of that organism.[17]

The blue mussel, *Mytilus edulis*, is an economically and ecologically important species in coastal and estuarine habitats globally, acting as a food source for humans[18] and creating complex habitats that offer refuge and protection to associated species.[19] It has also received particular attention with respect to OA studies[20] and metabolomics investigations.[21−23] However, little is known of the response of this marine bivalve exposed to a combination of environmental costressors using this approach. Consequently, we used ^{1}H NMR spectroscopy-based metabolomics to compare the response profile of mantle tissues of male and female blue mussels challenged with a combined exposure to reduced seawater pH, increased temperature, and the pathogen, *Vibrio tubiashii*. The aim was to elucidate whether these stressors impact specific biochemical pathways, how they act in combination and to what extent the responses observed are specific or are more general in nature. Five pH levels were tested: pH 8.05 (present day ambient seawater), pH 7.80 (IS92 emissions scenario, 2100 projection[3]), pH 7.60 (A2 scenario, 2100 projection[24]), pH 7.35 (IS92 scenario, 2300 projection[3]), and pH 6.50 (Carbon Capture Storage leak under acidified conditions[25]) and two different temperatures: 12.5 ± 0.5 °C (ambient seasonal temperature; western channel observatory at station L4) or 17.0 ± 0.5 °C (2100 warming scenario[3,26]). *V. tubiashii* was chosen as a pathogen due to its pathogenicity to bivalves and its link to a reduction in bivalve hatchery production.[7]

EXPERIMENTAL METHODS

Organism Collection and Exposure. Adult *Mytilus edulis* were collected from an intertidal estuarine mussel bed, Exmouth, U.K., during December. Mussels were transported to the laboratory within 2 h, cleared of all epibionts, and divided between 60 flow-through experimental chambers (4 mussels per chamber; vol = 250 mL). Chambers were continuously supplied with filtered seawater from 1 of 10 header tanks (vol = 450 L).

Header tanks contained natural seawater. The pH of the water was adjusted using the computerized feedback mechanism described by Widdicombe and Needham.[27] Header tank pH_{NBS} was maintained at one of five nominal pH levels: 8.05 (control), 7.80, 7.60, 7.35, and 6.50. Overflow from experimental chambers was runoff, creating a flow-through system preventing the accumulation of metabolic waste products. Each header tank supplied 6 replicate experimental chambers haphazardly distributed between 10 recirculating water baths (vol = 75 l; 125 × 60 × 10 cm). Water baths were maintained at either 12.5 ± 0.5 °C or 17.0 ± 0.5 °C, creating a fully crossed experimental design. Mussels were fed with *Isochrysis galbana*, dosed into header tanks daily to maintain an algal concentration between 100000 and 150000 cells ml^{-1}. Experimental chambers were subject to a 9:15 h light:dark regime.

Key environmental parameters, namely pH_{NBS} (Mettler Toledo InLab 413 SG), temperature, and salinity (Tetra con 325), were measured 3 times a week in seawater in the header tanks and experimental chambers. Total alkalinity (A_T) was measured using the open-cell potentiometric titration technique (Apollo Sci Tech model AS-ALK2) every 7 days, as described by ref 28. The remaining carbonate system parameters were calculated using CO_2SYS,[29] according to ref 30 (see Table 1).

Sampling Protocol and Bacterial Inoculation. Mussels were maintained as described above for 90 d, after which an individual was removed from each chamber to measure the metabolite profile of mantle tissue. On day 91 the posterior adductor muscle of remaining individuals was injected with 1.0 mL of a live suspension of *Vibrio tubiashii* NCIMB 1337 (ATCC19106; 2 × 10^6 bacterial cells mL^{-1}) . Mussels were emersed at their respective experimental temperatures for 2 h, held tightly shut with an elastic band to ensure sufficient bacterial distribution,[31] after which the band was removed and they were returned to the system. The mussel metabolome was then measured in one individual from each chamber on days 92 (1 day post-inoculation) and 98 (7 days post-inoculation).

Sex Determination. Mussel sex was assessed histologically by excising soft tissues from shells and taking a transverse slice through the mantle tissue. Tissues were processed following the protocol of ref 32. Dried sections were treated with Papanicolaou's stain,[33] which colors male reproductive tissues blue and female reproductive tissues mauve.[34] Where the sex of an individual could not be reliably determined (e.g., due to the absence of any gametes), individuals were classified as unsexed (11 of 144 organisms tested) and not included in subsequent analyses. Of the remaining 133 mussels, 74 were classified as males (27 sampled pre-exposure, 26 sampled 1 day post-inoculation, and 21 sampled 7 days post-inoculation) and 59 as females (22 sampled pre-exposure, 14 sampled 1 day post-inoculation and 23 sampled 7 days post-inoculation).

NMR Metabolomics. To sample the mussel mantle for metabolomics (3 out of 4 mussels sampled per experimental chamber; total 144 mussels), an additional transverse slice of mantle tissue was excised and immediately snap frozen in liquid nitrogen. Samples were stored at −80 °C until they could be analyzed. Polar metabolites were subsequently extracted from mantle tissue using the methanol/chloroform extraction method,[35] following Hines et al.,[17] with the exception of the use of a bead-based homogenizer (Precellys24). Immediately prior to analysis, dried polar extracts were resuspended in 0.1 M sodium phosphate buffer before being analyzed on a DRX-500 NMR spectrophotometer as described by Hines et al.[36] The polar metabolites were analyzed using one-dimensional ^{1}H NMR, using excitation sculpting for water suppression[37] and two-dimensional ^{1}H,^{1}H *J*-resolved (JRES) NMR spectroscopy, according to ref 38. Data were exported as the one-dimensional (1D) skyline projections of JRES spectra (pJRES) and converted to a format for multivariate analysis using custom-written ProMetab in MATLAB (version 7.1; The MathsWorks, Natick, MA[39]).

Spectral Preprocessing and Statistical Analysis. Each spectrum was segmented into 0.005 ppm bins between 0.6 and 10.0 ppm, with bins resulting from water and TMSP excluded from all spectra and data points between 7.988 and 8.016 ppm compressed into a single point. Data were normalized using the

Table 1. Carbonate Chemistry of Sea Water in (A) Header Tanks and Experimental Chambers Maintained at (B) 12.5 °C and (C) 17.0 °C for Each pH Exposure Level[a]

(A) parameter	8.05	7.80	7.60	7.35	6.50
pH_{NBS}	8.09 ± 0.01[a]	7.77 ± 0.01[b]	7.60 ± 0.01[c]	7.33 ± 0.01[d]	6.46 ± 0.02[e]
Temperature (°C)	13.8 ± 0.11	13.9 ± 0.10	13.9 ± 0.10	14.0 ± 0.10	13.9 ± 0.10
Salinity	34.2 ± 0.07	34.1 ± 0.07	34.2 ± 0.07	34.2 ± 0.07	34.2 ± 0.07
A_T (μmol/kgSW)	2402.3 ± 30.3	2396.7 ± 27.9	2412.7 ± 30.8	2392.4 ± 31.7	2420.7 ± 30.6
pCO_2 (μatm)[b]	518.0 ± 22.3[a]	1177.9 ± 64.1[b]	1940.4 ± 240.5[c]	5075.4 ± 1402.8[d]	25242.1 ± 1938.8[e]
$\Omega_{Calcite}$[b]	3.20 ± 0.08[a]	1.62 ± 0.06[b]	1.10 ± 0.08[c]	0.54 ± 0.09[d]	0.09 ± 0.01[e]
$\Omega_{Aragonite}$[b]	2.05 ± 0.05[a]	1.04 ± 0.04[b]	0.70 ± 0.05[c]	0.34 ± 0.06[d]	0.06 ± 0.01[e]
(B) parameter	8.05	7.80	7.60	7.35	6.50
pH_{NBS}	8.02 ± 0.01[a]	7.68 ± 0.01[b]	7.51 ± 0.01[c]	7.30 ± 0.01[d]	6.55 ± 0.01[e]
temperature (°C)	12.4 ± 0.09	12.3 ± 0.10	12.3 ± 0.10	12.3 ± 0.09	12.5 ± 0.10
salinity	34.0 ± 0.06	34.0 ± 0.06	34.0 ± 0.06	34.0 ± 0.06	34.0 ± 0.06
A_T (μmol/kgSW)	2395.2 ± 20.6[a]	2394.4 ± 20.0[a]	2436.4 ± 19.6[a]	2434.8 ± 17.5[a]	2584.8 ± 44.1[b]
pCO_2 (μatm)[b]	787.9 ± 30.6[a]	1704.9 ± 46.7[b]	2413.1 ± 92.9[c]	3786.1 ± 174.1[d]	24751.2 ± 1109.1[e]
$\Omega_{Calcite}$[b]	2.60 ± 0.08[a]	1.34 ± 0.04[b]	1.04 ± 0.06[c]	0.68 ± 0.03[d]	0.12 ± 0.01[e]
$\Omega_{Aragonite}$[b]	1.68 ± 0.05[a]	0.86 ± 0.03[b]	0.67 ± 0.04[c]	0.44 ± 0.02[d]	0.08 ± 0.01[e]
(C) parameter	8.05	7.80	7.60	7.35	6.50
pH_{NBS}	7.95 ± 0.01[a]	7.63 ± 0.01[b]	7.51 ± 0.01[c]	7.29 ± 0.01[d]	6.51 ± 0.01[e]
temperature (°C)	17.1 ± 0.07	17.0 ± 0.07	17.1 ± 0.07	17.2 ± 0.06	17.1 ± 0.08
salinity	34.1 ± 0.06	34.0 ± 0.06	34.1 ± 0.06	34.0 ± 0.06	34.0 ± 0.06
A_T (μmol/kgSW)	2407.4 ± 17.6[a]	2408.3 ± 18.3[a]	2424.3 ± 12.1[a]	2440.2 ± 17.6[a]	2630.1 ± 36.2[b]
pCO_2 (μatm)[b]	624.6 ± 21.8[a]	1535.2 ± 77.3[b]	2341.7 ± 104.8[c]	3494.1 ± 148.6[d]	21846.3 ± 1055.3[e]
$\Omega_{calcite}$[b]	2.64 ± 0.09[a]	1.24 ± 0.06[b]	0.85 ± 0.03[c]	0.59 ± 0.02[d]	0.12 ± 0.01[e]
$\Omega_{aragonite}$[b]	1.68 ± 0.06[a]	0.79 ± 0.04[b]	0.54 ± 0.02[c]	0.37 ± 0.01[d]	0.07 ± 0.01[e]

[a]Data are presented as mean (± S.E.). Significant differences ($p \leq 0.05$) between treatment levels, as tested by PERMANOVA, are indicated by different letters based on pair-wise tests. [b]Calculated using CO_2SYS.

Probabilistic Quotient approach and noise filtered, with the noise threshold set to 3 times the standard deviation of a region of known noise (9.5−10.0 ppm). This produced a data matrix of 144 samples by 1000 bins. This matrix was then subject to a generalized log transformation using the lambda parameter 1.49 e^{-9}, which stabilized the technical variance across the bins.[40,41]

Data were analyzed using the PERMANOVA+ add in[42] in PRIMER 6.1.[43] Data were first tested for homogeneity of variance and transformed if required. Euclidean distance similarity matrices were constructed, with p values calculated using 999 permutations of the residuals under a reduced model. Pair-wise comparisons were undertaken where a significant main effect, or an interaction between factors, was shown. Ordination of samples using nonmetric multidimensional scaling (MDS) was performed to display the biological relationships between samples, highlighting sample relatedness and, subsequently, sample grouping, in low-dimensional ordination space.[44]

Where a significant main effect was detected, data were further tested using SIMPER analysis. SIMPER analysis determines the percentage contribution of individual variables to overall group dissimilarity. Annotation of the NMR peaks shown to contribute over 50% of the group dissimilarity was undertaken, using in-house custom written software (Byrne et al., unpublished).

RESULTS AND DISCUSSION

Metabolite Composition of Mantle Tissue in Male and Female Mussels. A representative 1D projection of two-dimensional (2D) JRES NMR spectra of mantle tissue from male and female *M. edulis* is presented in Figure 1. Several metabolite classes were identified, including amino acids, organic osmolytes, and Krebs cycle intermediates. As shown previously for bivalves,[17,45−47] NMR spectra of both male and female mussels were dominated by betaine and taurine, shown to have 10−100 times greater intensity than other metabolites.

In agreement with previous research,[17,48] sex had a significant impact on the mussel metabolome (Pseudo-F_1 = 16.9, p = less than 0.001), as clearly demonstrated by MDS ordination (Figure 1c). However, despite supporting Hines et al.[17] in showing sex-related differences, the suite of metabolites that produced the sex difference was markedly different between these two studies. Hines and colleagues identified seven metabolites that were primarily responsible for the difference between male and female mussels (*Mytilus galloprovincialis*), with males characterized by higher concentrations of glycine, phosphoarginine, and glutamate, whereas females exhibited higher levels of acetoacetate, lysine, tyrosine and an unidentified metabolite at 3.69 ppm. In contrast, the present study identified 25 metabolites that contributed >50% of the dissimilarity between male and female *M. edulis* (Table S1 of the Supporting Information). Of these 25 metabolites, only 3 (glycine, glutamate, and tyrosine) were identified as discriminating for sex in both studies, with only glycine and tyrosine characterized as having similar male/female concentration ratios. The present study therefore highlights the significant biochemical differences between the sexes in *M. edulis* mantle tissue, which is perhaps not surprising given the role of this

dx.doi.org/10.1021/es501601w | Environ. Sci. Technol. XXXX, XXX, XXX−XXX

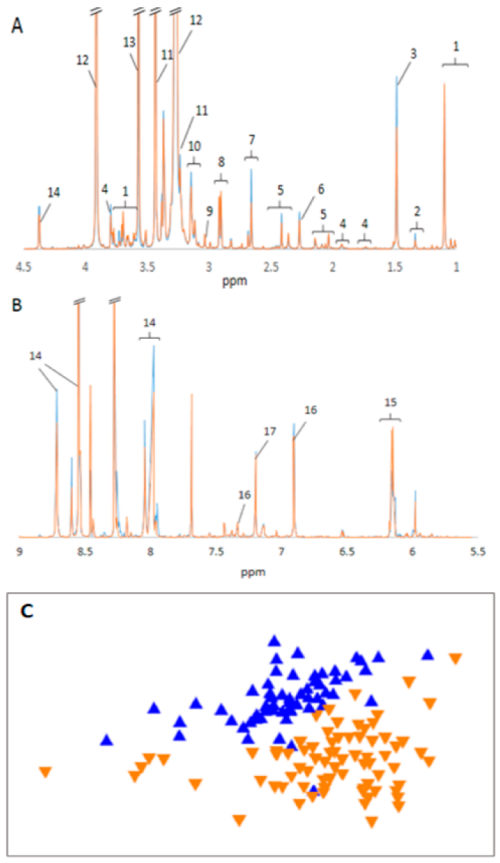


Figure 1. (A) Representative 1D projection of a 2D JRES NMR spectra of polar metabolites extracted from mantle tissues of male and female *M. edulis* under ambient (control) conditions, and (B) vertical expansion of the aromatic region of the NMR spectrum under the same conditions (male spectrum is represented by the orange line, female spectrum by the blue line). Keys: (1) Branched chain amino acids: isoleucine, leucine, and valine, (2) lactate, (3) alanine, (4) arganine, (5) glutamate, (6) succinate, (7) aspartate, (8) asparagines, (9) lysine, (10) malonate, (11) taurine, (12) betaine, (13) glycine, (14) homarine, (15) ATP/ADP, (16) tyrosine, and (17) histidine). (C) Nonmetric multidimensional scaling ordination plot for the Euclidean distance similarity metabolite data for sex (blue ▲ = females; orange ▼ = males).

tissue for gametogenesis.[34] Therefore, as suggested by Hines et al.[17] the differences between males and females are likely to originate from differences between egg and sperm associated biochemical signatures.

It is likely the differences between the mantle profiles of males and females identified by the present study and Hines et al.[17] were due to species differences or sampling season. Hines et al.[17] sampled two populations of mussels identified as *M. galloprovincialis*, between April and July. Conversely, the population in the present study, previously identified as *M. edulis*,[49] was collected in December and maintained until late March. By sampling in July, typically a period in the middle of the mussel spawning season, it is likely that Hines and colleagues[17] would have sampled ripe adults as well as spent organisms. However, exposing mussels between December and March, a period during which mussels typically reconstitute energy reserves before undergoing gametogenesis,[34] the present study design ensured all individuals were ripe and at the same reproductive state. By investigating the potential use of metabolomics for sex determination, a subsequent study by Hines and colleagues[48] demonstrated a similar impact of both species and spawning period on the mussel metabolome. While ripe males and females of *M. edulis* were characterized by a different suite of metabolites to *M. galloprovincialis*, spawning in *M. edulis* led to a subsequent shift in the profile of metabolites identified as discriminating for sex. This led these authors to caution the use of specific metabolites in sex determination and also highlighted the importance of accounting for reproductive state, species, and sex when assessing biomarkers of stress from metabolomic studies, a point supported by the present study.

Effect of Reduced pH on the Mussel Metabolome. In showing a clear separation between the control and elevated CO_2 exposed groups, this study demonstrated that elevated seawater CO_2 concentration significantly affected the metabolic profile of both male (Pseudo-F_4 = 1.62, p = 0.013) and female (Pseudo-F_4 = 1.65, p = 0.019) mussels. However, this effect was detectable only in mussels exposed to pH 6.50 in both groups, suggesting that mussels used in this current study were resilient to a level of seawater acidification projected to occur over the next 100−300 years. In highlighting the resilience of mussels, as indicated by the mussel metabolome, this study agrees with previous ocean acidification research involving this species.[50−52] The mussels used here were collected from an intertidal estuarine mussel bed. Such habitats frequently experience CO_2 concentrations significantly greater than expected from equilibrium with the atmosphere.[53,54] Therefore, it is possible the mussels used in this experiment were exapted to coping with reduced seawater pH. Thomsen et al.[51] demonstrated a similar resilience to reduced seawater pH in a population of mussels from Kiel fjord, where despite encountering seawater pH values <7.5 seasonally, individuals were able to maintain calcification, somatic growth, and juvenile recruitment.

While tolerant to moderate acidification, both males and females were impacted by significantly elevated CO_2. Both pairwise analysis and MDS ordination (Figure 2a) indicated that only males exposed to pH 6.50 were significantly different from the other pH exposure groups. Fifteen metabolites contributed >50% of this group dissimilarity in males, with this difference due to an alteration in the concentration of important osmolytes in the mantle tissue (Table S2 of the Supporting Information). By highlighting the role of organic osmolytes in characterizing the impact of reduced seawater pH, the present study supports[15] that a proposed disturbance of intracellular iso-osmotic regulation is the predominant mechanism by which elevated seawater pCO_2 impacts the metabolism of marine invertebrates. The free amino acid pool is crucial for balancing intracellular osmolality in stressed organisms.[39] Previous research in bivalves using metabolomics has shown that temperature,[55] hypoxia,[17,56] metal contamination,[45−47,57] pesticides,[47,56] and starvation[55,56] all impact the concentration of these organic osmolytes and amino acids in exposed samples, suggesting that a disturbance of osmotic balance may in fact be a generic response to, or biomarker of, environmental stress in these marine invertebrates. However, while vital for regulating osmolality, these oxidizable amino acids are also used in energy metabolism.[10,58] It is therefore possible exposure to pH 6.50 also results in disturbance to energy metabolism in males.

The idea that reduced seawater pH impacts energy metabolism is further supported by the increase in alanine, together with increased succinic acid (succinate), in males exposed to pH 6.50. Alanine is the major end-product in the anaerobic breakdown of glucose in bivalves.[57,59] Its presence,

dx.doi.org/10.1021/es501601w | *Environ. Sci. Technol.* XXXX, XXX, XXX−XXX

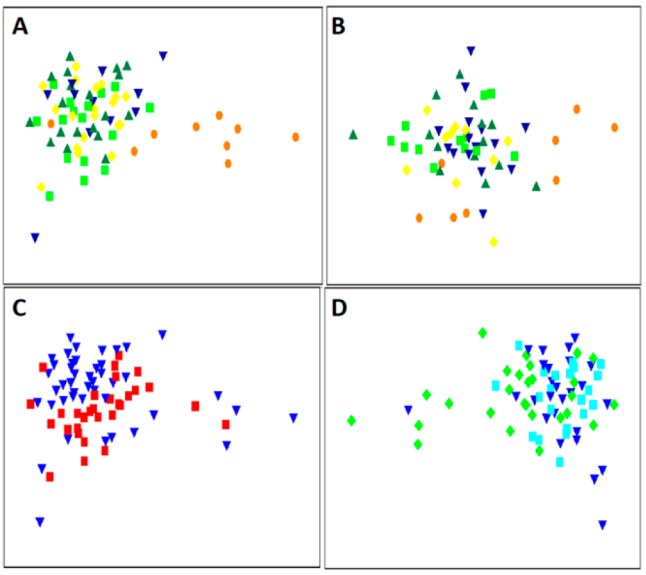


Figure 2. Nonmetric multidimensional scaling ordination plots for the Euclidean distance similarity metabolite data of (A) male mussels exposed to reduced seawater pH (black ▼ = pH 8.05; green ▲ = pH 7.80; green ■ = pH 7.60; yellow ♦ = pH 7.35; and orange ● = pH 6.50), (B) female mussels exposed to reduced seawater pH (black ▼ = pH 8.05; green ▲ = pH 7.80; green ■ = pH 7.60; yellow ♦ = pH 7.35; and orange ● = pH 6.50), (C) males exposed to increased temperature (blue ▼ = 12.5 °C and red ■ = 17.0 °C), and (D) males exposed to a bacterial exposure (green ♦ = pre-exposure, blue ▼ = 1 d post-inoculation, blue ■ = 7 d post-inoculation).

preceding an accumulation of succinate, is an early indicator of acute anaerobiosis.[60−63] Demonstrating a marked elevation of alanine and succinate in males exposed to pH 6.50, therefore suggests these organisms may have exceeded their optimum range for aerobic performance,[64] supporting Lannig et al.[14] who showed that an exposure to elevated CO_2 leads to an accumulation of succinate in both the gill and hepatopancreas of oysters. However, Lannig and colleagues[14] also measured a concomitant reduction in hemolymph oxygen concentration at a constant standard metabolic rate in exposed oysters, suggesting the operation of anaerobiosis. Because metabolic rate was not measured in the present study, we cannot confirm a reduced aerobic performance. Furthermore, the extent to which this anaerobiosis is a whole organism phenomenon or is restricted to the mantle tissue also needs elucidating.

In contrast to males, while exposure to pH 6.50 was also shown to significantly impact the metabolome of females, pairwise analysis indicated that individuals at this pH were only significantly different to those maintained at pH 8.05 and pH 7.80, with this group separation not apparent from MDS ordination (Figure 2b). Furthermore, 22 metabolites were identified as contributing over 50% of the dissimilarity between the females in the control group (pH 8.05 and 7.80) and those maintained at pH 6.50 (Table S2 of the Supporting Information). In highlighting many more metabolites that contribute to the pH group dissimilarity in females, it would appear that females were impacted differently to males, with a wider suite of biochemical processes being impacted in females rather than fewer shifts of greater magnitude as noted in males. This difference will therefore significantly impact the perceived sensitivity of mussels to reduced seawater pH, with accounting for sex-differences vital for accurately interpreting metabolic data as a biomarker of reduced seawater pH.

As with males, exposure to low pH resulted in a significant alteration in the concentration of organic osmolytes and amino acids in the mantle tissues of female mussels. Exposure to pH 6.50 led to an increase in alanine, proline, isoleucine, leucine and tyrosine, while aspartate was demonstrated to decrease in low pH. This was the same response as noted in males at this elevated CO_2 concentration. Conversely to males, however, the concentration of valine, glutamine, and threonine are all reduced, and glycine increased, in females at pH 6.50. Furthermore, while an impacted energy metabolism is indicated via an increase in the concentration of succinate in males maintained at pH 6.50, in females, the concentration of succinate is reduced at this pH. As succinate is a Kreb's cycle intermediate, a reduction in this metabolite may in fact indicate increased aerobic metabolism rather than reduced aerobic scope in these mussels. This would also appear to be supported by the decrease in adenosine monophosphate (AMP). Bundy et al.,[65] in exposing the earthworm *Lumbricus rubellus* to copper, proposed that AMP and adenosine diphosphate (ADP) were inversely correlated to adenosine triphosphate (ATP), with an increase in AMP and ADP, indicating disturbed energy metabolism. However, in noting a decrease in AMP at pH 6.50, the opposite would appear to be the case in female mussels, with enhanced energy metabolism at this pH. However, as with males, the nature and extent of this phenomenon requires further investigation. Furthermore, while exposure to pH 6.50 is shown to impact the metabolome of both male and female mussels, it is important to consider this result in light of the increased mortality experienced in this group (Table S4 of the Supporting Information). By noting higher mortality at this pH, the resulting metabolomic data from males and females at pH 6.50 is that of the surviving population, which may impact the perceived sensitivity of organisms to this exposure level.

Effect of Temperature on the Mussel Metabolome. An increase in environmental temperature results initially in an increase in metabolism in ectothermic organisms.[66] Whether or not the increase in metabolism is sustained in the medium to long-term, there are likely to be resultant changes in that metabolism. So demonstrating a temperature effect on the mussel metabolome is perhaps not surprising. Therefore, it is interesting that a temperature effect was only detectable in males (Pseudo-F_1 = 2.07, p = 0.015), with the female metabolome showing no significant effect of warming (Pseudo-F_1 = 1.14, p = 0.297). To our knowledge, this is the first study using metabolomics that has demonstrated such a contrasting response of males and females of a marine invertebrate species to anthropogenic climate change. Such a finding, if it generalizes, will have significant consequences for mussel population dynamics under future climate change conditions, as well as for the interpretation of metabolomic data as a biomarker of this abiotic stressor.

While temperature impacted the metabolome of male mussels, MDS ordination revealed only minimal temperature-related separation (Figure 2c). Twenty-two metabolites were responsible for >50% of this group dissimilarity, with a decrease noted in 17 of these metabolites (Table S2 of the Supporting Information). As with reduced seawater pH, it was a change in the abundance of organic osmolytes and amino acids that contributed most to the dissimilarity between males maintained at 12.5 and 17.0 °C, again suggesting a general stress-induced impact on iso-osmotic regulation or energy metabolism in these bivalves.[10,58]

dx.doi.org/10.1021/es501601w | *Environ. Sci. Technol.* XXXX, XXX, XXX−XXX

Identifying alanine and succinate to be important factors in the separation of the control and increased temperature mussel groups further demonstrates the importance of temperature for energy metabolism in male mussels. However, in contrast to males exposed to pH 6.50, alanine decreased under a warming scenario. Additionally, succinate only marginally increased, suggesting little recourse to anaerobiosis over this temperature increase. Indeed, as alanine can be transaminated to pyruvate as part of gluconeogenesis, a process that fuels an increased metabolic demand,[14] recording a decrease in alanine more likely points to an increase in aerobic metabolism. Enhanced aerobic metabolism in males under increased temperature is also supported by the increase in AMP at 17.0 °C. Showing an altered energy metabolism in males exposed to a warming scenario therefore suggests this abiotic factor will be important in determining male mussel performance under warming conditions over the coming century.[3]

Effects of a Pathogenic Challenge on the Mussel Metabolome. Exposure to a pathogenic challenge is thought to be energetically costly.[67] Therefore, it is not surprising that bacterial exposure had a significant effect on the mussel metabolome. As with temperature, however, this effect was demonstrated only in males (Pseudo-F_2 = 2.27, p = 0.003), with the metabolome of females seemingly unaffected by bacterial inoculation (Pseudo-F_2 = 1.31, p = 0.177). Pair-wise analysis indicated that the metabolome of males measured preinoculation and 1 d post-inoculation were significantly different to mussels measured 7 d post-inoculation. Yet this sample separation was not clear from the MDS ordination (Figure 2d). Furthermore, there was no significant difference between mussels sampled pre-exposure and 1 d post-inoculation. Twenty-one metabolites were responsible for >50% of the group dissimilarity between pre-exposure and 1 d post-inoculation males compared to 7 d post-inoculation males (Table S2 of the Supporting Information).

As with both reduced seawater pH and elevated temperature in males, the predominant mechanism by which a bacterial exposure impacts these organisms is through disturbance of isosmotic balance and energy metabolism. The occurrence of these disturbances, irrespective of the stress imposed, would suggest that these processes may act as generic biomarkers of environmental stress in bivalves. However, with the response of individual metabolites, and the magnitude of change in each metabolite, being specific to each stressor, it would appear these processes are affected differently by pH, temperature, and a bacterial inoculation. As with temperature, the metabolite contributing most significantly to the difference between mussels measured pre-exposure and 1 d postexposure compared to those sampled 7 d after an exposure to *Vibrio tubiashii* was alanine. A reduction in alanine may indicate an increased energetic demand in infected mussels and thus an increased metabolic rate. However, this was present only in males sampled after 7 days. Therefore, it would appear the energetic constraint of a bacterial inoculation takes time to reach a detectable level. Additionally, a pathogen exposure was shown to impact formic acid and an unidentified metabolite at 1.10 ppm. While the identity of the spectral peak at 1.10 ppm, as well as the mode of action of these two metabolites, remains unknown, these metabolites were also impacted by temperature in males, as well as reduced pH in females (Table S2 of the Supporting Information). Furthermore, the unidentified metabolite at 1.10 ppm was also present in mussels exposed to cadmium.[46] It is suggested that this unidentified metabolite may be important for characterizing the stress response of mussels. Thus, elucidating the identity and nature of these metabolites is crucial to further understanding on the impact of environmental stress in mussels.

Metabolic Response of Mussels Exposed to Combined Stressors. In addition to significant main effects of both reduced seawater pH and bacterial exposure in males, there was also a significant interaction between the two experimental factors (Pseudo-F_8 = 1.47, d.f. = 8, p = 0.018). Pairwise analysis and MDS ordination (Figure 3a) show that prior to a bacterial exposure, the metabolome of mussels exposed to pH 6.50 was significantly different from all other treatment groups, whereas mussels exposed to pH 7.35 were also significantly separated from the control group (pH 8.05) and mussels exposed to pH 7.80. Conversely, in males measured 1 d post-inoculation, mussels maintained at pH 7.80 were significantly separated from those maintained at pH 7.60 and pH 6.50; however, this pattern was not detectable from MDS ordination (Figure 3b). Furthermore, there were no significance differences between any of the other pH treatment. By 7 d post-inoculation, there was no significant difference in the metabolome of mussels exposed to any of the pH groups, as indicated by MDS ordination (Figure 3c).

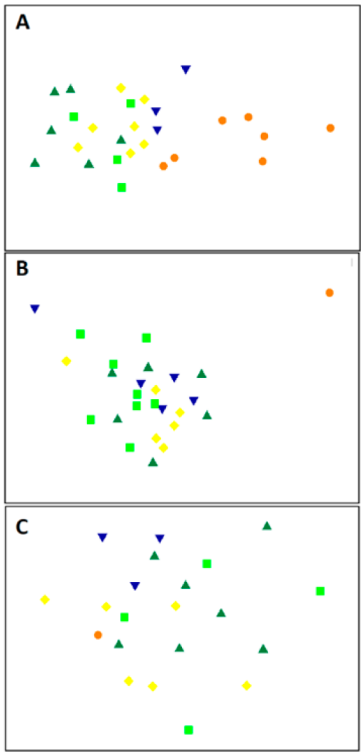


Figure 3. Nonmetric multidimensional scaling ordination plots for the Euclidean distance similarity metabolite data of male mussels exposed to reduced seawater pH (black ▼ = pH 8.05; green ▲ = pH 7.80, green ■ = pH 7.60; yellow ♦ = pH 7.35, orange ● = pH 6.50) prior to a (A) bacterial exposure, (B) 1 day post-inoculation, and (C) 7 days post-inoculation.

The significant interaction between reduced seawater pH and an exposure to *V. tubiashii* demonstrates the potential impact of environmental costressors on organism energy homeostasis. Prior to a bacterial exposure, mussels exposed to pH 6.50 were characterized by alterations in organic osmolytes and amino acids, as well as metabolites that indicate a disturbance of

energy metabolism accompanying the onset of anaerobiosis (Table S3 of the Supporting Information), as was also seen from the main effect of reduced seawater pH in this group. However, in addition, mussels exposed to pH 7.35 were identified as being significantly different from the control group (pH 8.05 and 7.80). It could be that male mussels sampled prior to a bacterial exposure were more sensitive to reduced seawater pH than was suggested by the main effect of reduced seawater pH alone. Furthermore, in addition to disturbed osmotic balance in this group, this dissimilarity was predominantly characterized by a decrease in alanine, suggesting elevated aerobic metabolism, rather than the onset of anaerobiosis. Increased metabolic rate in mussels exposed to moderate seawater acidification (pH 7.70, 7.38, and 7.14) was also found by ref 52 with increased rates of oxygen uptake suggested to compensate for increased cellular energy demand and increased nitrogen loss under these conditions. As environmental stressors seldom occur in isolation in nature,[5] measuring a different sensitivity to reduced seawater pH in mussels from a combined stressor exposure, compared to the main effect of this stressor, the present study highlights the need for metabolomics studies to account for multiple stressors and for stressor interactions when determining biomarkers of stress.

Pre-exposure, male mussels were significantly affected by reduced seawater pH, yet exposure to a pathogen was shown to reduce the dissimilarity between mussels at the different pH levels, to the extent that by 7 d post-inoculation, there was no longer a significant impact of seawater pH on the male metabolome. Furthermore, at 1 d post-inoculation, males exposed to pH 6.5 were only significantly different from those at pH 7.80 (Table S3 of the Supporting Information). Environmental stress impacts energy homeostasis, in turn affecting the allocation of energy to competing physiological processes.[68] Prior to a bacterial exposure, reduced seawater pH impacted energy metabolism, as indicated by the onset of anerobiosis in the pH 6.50 group, and enhanced aerobic metabolism at pH 7.35. However, it is possible the additional energetic burden of a pathogenic challenge in organisms measured post-inoculation, particularly 7 d post-inoculation, may counteract this energy based pH dissimilarity, resulting in a convergence of samples. Therefore, when measured using metabolomics, this results in the absence of a significant effect of pH at 7 d post-inoculation, despite the organisms in all pH treatments likely experiencing environmental stress.

While pH and bacterial exposure were shown to interact in male mussels, there was no further interaction between experimental factors affecting the male metabolome. Furthermore, while seawater pH was shown to impact the metabolome of female mussels independently, there was no significant interaction between any of the main experimental factors in female mussels.

As suggested by the response of the metabolome, exposure to reduced seawater pH, increased temperature and a pathogen exposure appear to impact the energy metabolism and osmotic balance of the blue mussel, *Mytilus edulis*; however, this appears to be through a generalized stress response. Furthermore, males and females are shown to respond differently to environmental stressors. This finding subsequently has important implications for the interpretation of metabolomic data in this group and of the impact of environmental stress in mussels.

■ ASSOCIATED CONTENT

Supporting Information

Tables presenting the percentage contribution of specific metabolites to the group dissimilarity, and the magnitude of metabolite changes, measured between male and female mussels, as well as in mussels exposed to reduced seawater pH, increased temperature, and a pathogen. Table outlining mortality in different treatment groups. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by a U.K. Natural Environmental Research Council (NERC), Ph.D. studentship awarded to R.P.E., as well as access for R.P.E. to work at the Biomolecular Analysis Facility at the University of Birmingham (R8-H10-61). This study was undertaken at PML using OA exposure facilities partly funded by a joint DEFRA/DTI funded project (IMCO2) and the NERC standard grant (NE/C510016/1) awarded to S.W.

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