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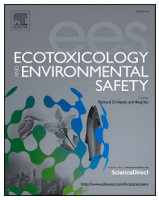

Lead exposure modulates thermal vulnerability in *Mytilus edulis* under multi-stressor conditions

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ABSTRACT

Studying the cumulative impacts of interacting stressors is essential for predicting ecosystem-wide risks from anthropogenic stressors, yet multi-stressor experiments remain scarce. Here, we investigated how lead (Pb) contamination influences survival and cross-tolerance to salinity and thermal stress in Arctic blue mussels (*Mytilus edulis*). Mussels were exposed to environmentally relevant Pb concentrations, followed by sequential exposure to varying salinities (5–25) and aerial temperatures (5–36 °C). Survival patterns were assessed, and transcriptomic responses were analysed using RNA sequencing. Heat-induced mortality occurred across treatments, and Pb exposure elevated baseline mortality. However, elevated tissue Pb concentrations significantly attenuated temperature-driven mortality, indicating partial cross-tolerance between contamination and thermal stress. Reduced salinity increased vulnerability to heat stress and weakened the protective effect of Pb. Transcriptomic analyses revealed upregulation of cellular stress-response pathways, including detoxification-related genes, consistent with transcriptional front-loading mechanisms underpinning cross-tolerance. Gene expression responses were modest overall, reflecting the high physiological plasticity of *Mytilus* mussels. Our findings demonstrate that pollution can alter thermal sensitivity in non-additive ways, complicating predictions of organismal responses to environmental changes. Thus, accounting for multi-stressor interactions is critical to understand species resilience in a rapidly changing world.

1. Introduction

The polar regions are warming faster than the global average, accelerating the melt of sea ice and glaciers and causing cascading effects in marine ecosystems (Meredith et al., 2019). Although there is a growing interest in understanding how climate change impacts polar species' performance and biodiversity, little focus has been given to the polar coastal zone, a complex multi-stressor environment where species are exposed to a mosaic of interacting physical, chemical, and biotic stressors of both marine and terrestrial origin (Sejr et al., 2024; Thyrring, 2025). Interacting stressors may affect species in non-additive ways, alleviating (antagonistic effects) or exacerbating (synergistic effects) the impacts of climate change (Crain et al., 2008). For example, adaptation to elevated CO_2 levels interacts antagonistically with warming on metabolism in marine diatoms (Lin et al., 2025), and decreasing salinity increases sensitivity to heat stress in intertidal mussels (Barrett et al., 2022). However, the broader impacts of interacting stressors are poorly understood in polar regions (Thyrring et al., 2025).

Foundation species provide habitats, promote biodiversity, and enhance ecosystem resilience. Thus, understanding the effects of multifaceted stressors on habitat-forming species is essential to understanding ecosystem-wide responses (Barra et al., 2025). Blue mussels from the *Mytilus edulis* species complex are foundation species in rocky intertidal ecosystems, where mussel beds support considerable biodiversity. Blue mussels are resilient to thermal stress (Clark et al., 2021; Thyrring et al., 2026), and increasing temperatures are predicted to facilitate their poleward expansion in the Arctic (Thyrring et al., 2017). However, exposure to other stressors associated with Arctic climate change may offset range expansion and cause blue mussel populations to decline (Thyrring et al., 2023). For instance, the ice melt from Greenland's Ice Sheet has increased sixfold since the 1980s in response to

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warming (Mouginot et al., 2019), and the resulting freshwater discharge creates salinity gradients within fjords, where surface salinity can drop below 5 near freshwater outlets (Meire et al., 2017). Low salinities increase the mortality of blue mussels exposed to 30 °C (Nielsen et al., 2021), a temperature below maximum intertidal summer temperatures in Greenland, which can exceed 36 °C (Sejr et al., 2021; Thyrring et al., 2017). Furthermore, Greenland has a long history of mineral exploration, and long-term environmental impacts from early mining operations include trace metal contamination of sediments and benthic biota (Johansen et al., 2008; Rigét et al., 1997). For example, lead (Pb) concentrations in blue mussels collected near decommissioned mining operations still exceed 1500 μg/g dw (Søndergaard et al., 2010), and concentrations are expected to remain elevated for decades to come (Søndergaard et al., 2011). The ecological impacts of elevated Pb contamination in Arctic coastal species remain largely unknown, but temperature, Pb contamination, and salinity influence shared physiological pathways (e.g., heat shock protein expression) and may interact in complex ways to affect survival and resilience to climate change. Considering the complex mosaic of stressors, intertidal species in polar coastal zones may be increasingly susceptible to the effects of climate change.

Most studies, however, do not consider physiological plasticity in climate change research, yet this often overlooked mechanism plays an important role in determining sensitivity to stress, especially in multi-stressor experiments, and it is important to consider it when predicting responses to environmental change (Clark et al., 2019; Seebacher et al., 2014). Plasticity occurs at multiple biological levels, from genes to whole animals, and is a well-known mechanism that enables species to cope with rapid environmental change. Plastic responses can lead to higher stress tolerance within or across generations (Donelson et al., 2017), and blue mussels exhibit highly plastic responses to tidal height and seasonal acclimation (Buckley et al., 2001; Chapple et al., 1998; Ioannou et al., 2009; Roberts et al., 1997). In some cases, adaptation to a given stressor enhances individuals' capacity to cope with another stressor, a phenomenon known as cross-tolerance (Collins et al., 2021; Todgham et al., 2005; Todgham and Stillman, 2013). Cross-tolerance may arise through preparatory mechanisms such as transcriptional front-loading, in which constitutive gene expression is adjusted following the initial stress exposure (Barshis et al., 2013; Gunderson et al., 2016). For example, preliminary exposure to sub-lethal heat stress has been shown to increase tolerance to hypoxic and hyperosmotic conditions in tidepool sculpins (Todgham et al., 2005), and parasite infections can offer protection against heat stress through the induction of heat shock proteins (Greve et al., 2026; Selbach et al., 2020). Laboratory studies often apply stressor combinations simultaneously, but sequential exposure studies are necessary to determine whether such preparative responses are induced by the initial stressor. If a secondary stressor (e.g., salinity) affects the same genes as the initial stressor (e.g., temperature), front-loading may provide cross-tolerance and enhanced protection. Thus, considering cross-tolerance is important for accurately predicting an organism's capacity to adjust to altered environments.

Therefore, this study uses environmentally realistic levels of Pb, salinity, and air temperatures to investigate how pollution, salinity, and temperature stress interact to affect survival and, using an RNA-seq approach, whether transcriptional front-loading generates cross-tolerance among stressors.

2. Materials and methods

2.1. Animal collection and holding conditions

Blue mussels were collected in August 2023 from a pure *Mytilus edulis* population (Mathiesen et al., 2017) in the intertidal zone of Kobbefjord near Nuuk, Greenland (64°N). Mussels were wrapped in wetted towels and transferred to aerated aquaria before transportation to holding facilities at Aarhus University, Denmark, within 48 h of collection. Mussels were distributed into ten 20 L aquaria of constant salinity (25) in a cold room (5 °C) and allowed to acclimate to laboratory conditions for eight days before any experiments were conducted. Half the water volume was exchanged daily, and mussels were afterwards fed a phytoplankton mix (ReedMariculture, Shellfish Diet—1800 Formula, Campbell, CA, USA). It should be noted that no mussels died during the acclimation period.

2.2. Experimental design

To investigate cross-tolerance and survival following exposure to salinity and thermal stress in blue mussels contaminated with lead (Pb), we designed a full factorial experiment with four air temperatures (5, 30, 33, and 36 °C), three salinities (5, 15, and 25), and five Pb levels (0, 0.4, 0.8, 1.6, and 3.2 mg/L), for a total of 60 treatments (Supplementary Fig. S1). A realistic range of Pb contamination levels (0–2000 μg Pb/g dw (dry weight)) was obtained by keeping mussels in aquaria with Pb-contaminated seawater (salinity of 25) with a nominal concentration of 0, 0.50, 1.80, 4.50, and 6.50 mg Pb/L for 10 days at 5 °C before starting the experiment. The Pb seawater was made by supplementing seawater with different lead concentrations (detailed above) using a stock solution of 10 g Pb/L prepared using the analytical salt lead(II) nitrate $[Pb(NO_3)_2]$ (Sigma-Aldrich Solutions) and deionized water (Thyrring et al., 2015). Pb seawater was replenished every 24 h, and mussels were daily exposed to air (5 °C) for 1.5 h to simulate low tide conditions.

After 10 days of Pb contamination, mussels were transferred to new aquaria (0 mg Pb/L) containing seawater of either 5, 15, or 25 of salinity for 24 h. To simulate aerial heat exposure, 13–14 mussels from each Pb x salinity combination were exposed daily, over the course of 4 days, to air for 1.5 h by removing mussels from the aquaria and transferring them to pre-heated climate chambers (5, 30, 33, or 36 °C) (Memmert GmbH, Germany). After 1.5 h, the mussels were transferred back to aquaria with seawater (salinity 25) for overnight recovery. It should be noted that animals were kept in seawater of salinity 25 throughout this final stage of the experiment. Every morning, dead mussels were removed, and the shell length was measured using a digital calliper. After the last (4th) air exposure cycle, the length of all remaining mussels was measured. Gill tissue was flash-frozen in liquid nitrogen and stored at −80 °C for analyses of Pb content and gene expression.

2.3. Pb analysis

The quantification of Pb in the tissue samples was performed using inductively coupled plasma mass spectrometry (ICP-MS). For the analysis, the flash-frozen samples were homogenised and freeze-dried, with approximately 0.05–0.2 g of each sample used for digestion. Then 1 mL of 65% HNO_3 and 2 mL of 30% H_2O_2 were added to each sample, and the acid digestion was performed in a CEM MARS 6 microwave. The temperature was increased to 180 °C over 25 min, then maintained at 180 °C for 5 min. After cooling, ultrapure water was added to each sample to reach a final volume of 25 mL. Quality control of digestion was ensured by using digested blanks, containing only the acid mixture. The Pb concentration in blanks was below the limit of quantification (LOQ; 0.1 μg/L).

2.4. Transcriptomics

To provide further detail on the multi-stressor responses, transcriptomic analyses were performed on a subset of the samples. For each sample chosen to take forward for RNA analyses, mortality rates were less than 8% or one death in a cohort of 13–14 (Supplementary Table S1). To dissect the differences between responses to Pb, salinity, and temperature, two transcriptomic experiments were performed. In the first, Experiment A, the responses to Pb (0.8 and 3.2 mg/L) were

evaluated at different salinities (5, 15, and 25) at the ambient temperature of 5 °C. In Experiment B, the responses to Pb (0.8 and 3.2 mg/L) at the two salinities of 15 and 25 were evaluated at 30°C (Supplementary Fig. S2). For both experiments, RNAs were extracted and sent for sequencing and bioinformatics analyses at Novogene, United Kingdom, using the same parameters (as described below), but with a *de novo* transcriptome generated for each experiment and the two experiments analyzed separately (see Supplementary Figure S2 for bioinformatics experimental design and sample abbreviations).

2.5. Extraction of RNA, library preparation, and sequencing

RNA from flash-frozen gill tissue was extracted using the Promega SV Total RNA kit according to the manufacturer's instructions, with the final elution in 55 μL DEPC-treated sterile distilled water. RNAs were quantified using a Qubit 4 fluorometer (Invitrogen Thermo Scientific). A quality check to ensure RNA integrity was performed by running eight randomly selected samples on an agarose gel. All samples passing the quality check were sent to Novogene for library preparation, sequencing, and bioinformatics analyses. For each library, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, cDNA was synthesized using random hexamers. Each library was quantified by Qubit and real-time PCR, and assessed by bioanalyzer for size distributions. PE150 libraries were run on an Illumina NovaSeq X Plus to produce ~6 Gb of data per sample.

2.6. Statistical analyses

2.6.1. Survival

Final-day survival (Day 4) was analyzed using a binomial generalized linear model (GLM) with a logit link function. Mortality status (dead/alive) was modeled as a function of Pb concentration (treated as a categorical experimental factor), salinity, temperature, and their two-way interactions. Model selection was based on Akaike's Information Criterion (AIC), and the final model included all two-way interaction terms. Statistical significance of predictors was assessed using Type II Wald χ^2 tests. Model assumptions were evaluated by inspecting residual diagnostics and testing for overdispersion. Survival over time was analyzed using Cox proportional hazards regression to evaluate the effects of Pb concentration, salinity, and temperature on mortality risk over time (Cox, 1972). The model included all main effects and their two-way interactions. Model assumptions were verified through standard diagnostic procedures: proportional hazards were assessed using Schoenfeld residuals (*cox.zph* function in the R package survival (Therneau, 2024; Therneau and Grambsch, 2000)), linearity of continuous covariates was examined via Martingale residuals, and influence diagnostics (DFBETA) confirmed that no individual observations disproportionately affected the results. Because the proportional hazards assumption was violated for temperature ($p < 0.001$), a time-varying Cox model was fitted by incorporating a temperature-dependent interaction term, allowing the effect of temperature on mortality hazard to change through time. The time-varying model provided a significantly better fit than the standard proportional model ($\Delta AIC = 13.4$; likelihood ratio test, $p < 0.05$) and satisfied all modal validation criteria. Survival was performed in R version 4.3.2 (R Core Team, 2025) using the packages survival and survminer (Kassambara et al., 2025). The final survival patterns were visualized using Kaplan–Meier survival curves and compared among treatments using log-rank tests.

2.6.2. Bioinformatic analysis

All bioinformatic analyses were performed by Novogene. Briefly, raw reads were cleaned by removing adapters and low-quality reads using fastp (Chen et al., 2018). Assembly was performed using Trinity (v2.15.1) (Haas et al., 2013), including the software modules Inchworm, Chrysalis, and Butterfly to generate a trinity.fasta file. Hierarchical clustering to remove redundancy was performed using Corset (Davidson and Oshlack, 2014), and the longest transcripts in each cluster were selected as unigenes. Benchmarking of the finalised transcriptome was performed using BUSCO (Simão et al., 2015). Annotation of transcripts was performed against seven databases, namely NR (NCBI non-redundant protein sequences), NT (NCBI nucleotide sequences), KO (KEGG Orthology), SwissProt (Manually annotated protein sequences), PFAM (Protein domains and families), now in InterPro, GO (Gene Ontology), and KOG (euKaryotic Orthologous Groups). For reference transcriptome alignment, all clean reads were mapped to the Trinity reference sequence transcriptome using RSEM (v1.3.3) (Li and Dewey, 2011) and bowtie2 (parameter of mismatch = 0) (Langmead and Salzberg, 2012). Those with a comparison value below 10 were filtered out along with unpaired reads and those mapping to multiple regions. Differential expression analysis was performed using DESeq2 (Love et al., 2014). The resulting *p*-value was adjusted for the false discovery rate using (Benjamini and Hochberg, 1995). The threshold of significant differential expression *p*-adj $<= 0.05$ and $|\log2(\text{fold change})| >= 1$. Gene Ontology enrichment of differentially expressed genes was carried out using the R package Goseq (Young et al., 2010), with corrected *p*-values < 0.05 considered significantly enriched.

3. Results

3.1. Pb content in mussels

There was a positive relationship between soft tissue Pb content and seawater Pb concentration (Fig. 1). The concentration of Pb in mussels increased with increasing Pb concentrations in the water until 1.6 mg/L, and the highest internal concentration was detected in mussels exposed to water Pb concentrations of 1.6 mg/L and 3.2 mg/L, with a mean tissue concentration of 1352.77 μg/g dw (dry weight) and 1481.25 μg/g dw in the 1.6 mg/L and 3.2 mg/L treatment, respectively. The Pb content in control mussels was 2.11 μg/g dw.

3.2. Survival and cross-tolerance

After four days of repeated air exposure, increasing Pb contamination levels (GLM; $\beta = 10.55 \pm 3.20$, $p = 0.001$) and air temperature (GLM; $\beta = 1.65 \pm 0.31$, $p < 0.001$) increased mortality (Fig. 2), while salinity had no effect (GLM; $\beta = -0.25 \pm 0.09$, $p = 0.57$). A significant negative interaction between Pb and temperature (GLM; $\beta = -0.26 \pm 0.09$, $p = 0.005$) indicates that the temperature-related increase in mortality risk was attenuated at higher Pb concentrations, suggesting partial cross-tolerance between Pb contamination and thermal stress.

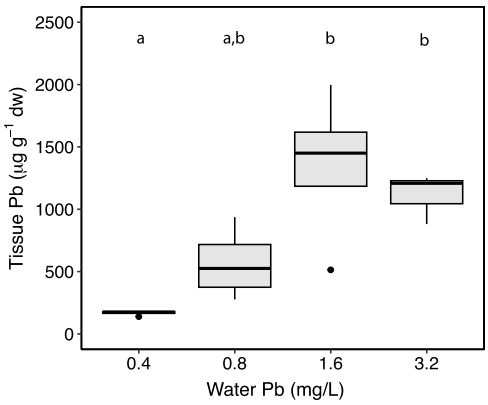

Fig. 1. Lead (Pb) concentrations (μg/g dw) in gills of *Mytilus edulis* exposed to four different Pb concentrations for 10 days. The horizontal line in each boxplot is the median, the boxes define the hinges (25–75% quartile), and the whisker is 1.5 times the hinges. Black dots represent data outside this range. Different letters indicate a significant difference ($p < 0.05$).

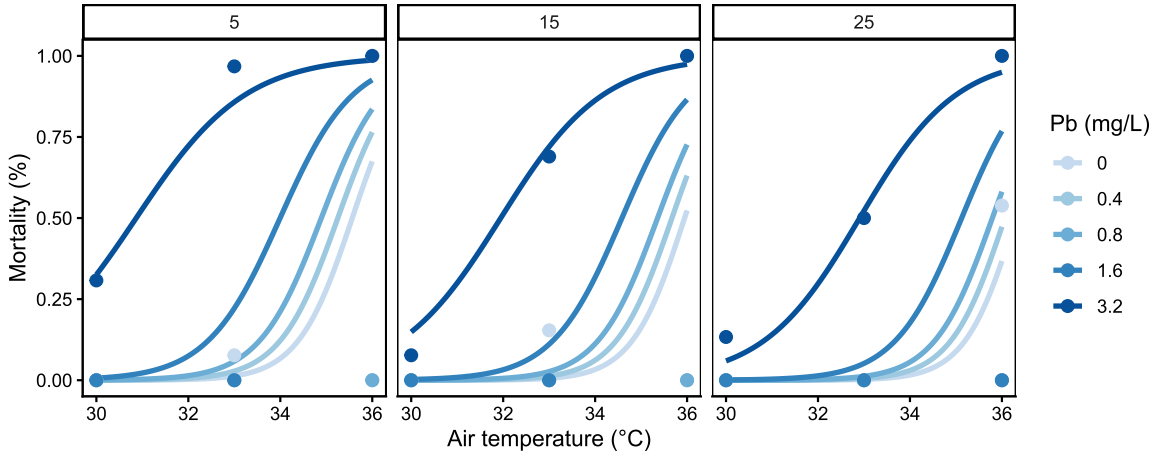

Fig. 2. Mortality after four consecutive days of repeated aerial exposure in Pb-contaminated *Mytilus edulis* exposed to three salinity levels (5, 15, and 25). Colored lines represent fitted lines for the five Pb exposure levels (0, 0.4, 0.8, 1.6, and 3.2 mg Pb/L) applied during a 10-day contamination phase preceding the experiment. Dots indicate actual survival.

The Cox proportional hazards model with time-varying temperature effects revealed differences in mortality risks of Pb-contaminated blue mussels exposed to various combinations of salinities over time. Survival declined progressively with repeated aerial exposures across all salinity treatments (Fig. 3). Mortality risk increased with increasing temperature ($\beta = 1.72 \pm 0.32$, HR = 5.59, $p < 0.001$) and decreasing salinities ($\beta = -0.45 \pm 0.17$, HR = 0.64, $p = 0.008$) (Fig. 3), and a negative time-varying term for temperature ($\beta\_tt(T) = -0.81 \pm 0.24$, HR = 0.44, $p < 0.001$) indicated that the relative influence of temperature on mortality risk declined with time as the incremental risk decreased over the course of the experiment (Supplementary Fig. S3). A significant interaction between Pb and salinity ($\beta = 0.010$, HR = 1.01, $p = 0.035$) demonstrated that the protective effect of high salinity levels against thermal stress diminished under increasing Pb exposure, and the interaction between salinity and temperature ($\beta = -0.012$, HR = 1.01, $p = 0.007$) indicates that elevated salinity slightly increased mussel resilience to high-air-temperature stress.

3.3. Transcriptome assemblies

Sequencing of 35 libraries for Experiment A produced a total of 1076,751,166 reads, whilst Experiment B comprised 29 libraries, which produced 920,162,306 reads. In both experiments, there was a 99% retention after cleaning and an error rate of 0.01% (see Supplementary File S1 for full statistics). Assembly of the sequencing data from Experiment A generated 367,600 unigenes, whilst the data from Experiment B produced 331,103 unigenes. The assembly statistics and metrics for each experiment were very similar, with annotation rates of ~63%, although this varied by database: 48% of unigenes matched entries in the NT database, but only ~10% matched entries in the Swissprot database (for full assembly statistics and metrics see Supplementary File S2).

3.4. Differential expression and GO enrichment

Mussel response to the different conditions in both experiments was relatively muted, limited to a few hundred differentially expressed genes at most. The number of up-regulated genes varied between 87 and 472, and down-regulated genes varied between 99 and 316 (Table 1). Unfortunately, as with most non-model species, annotation rates were low. Most of the *Mytilus* sequences annotated using the NCBI non-redundant protein sequences (which produced the highest annotation rates) as either "hypothetical predicted protein" or "predicted uncharacterised protein" with no functional annotation, as evidenced by the very low number of matches to the curated databases SwissProt and GO. It is these latter two databases, which can be used to infer biological processes affected by the different treatments, that are obviously difficult to use given such low annotation rates. Therefore, molecular analyses were centred on up-regulation, GO enrichment in Biological Process (BP) (Supplementary File S3), and the identification of heat shock proteins (HSPs) and stress proteins in the differentially expressed transcripts.

3.5. GO enrichment

Enriched GO categories in Experiments A, which was conducted at 5 °C and therefore comprised a dual stressor experiment of reduced salinity and Pb addition, generally concentrated on increasing energy metabolism (e.g., carbohydrate metabolic process and lipid metabolic process) in the low Pb levels (0.8 mg/L) at salinities of 15 and 25. These categories were replaced by more structural changes (cell differentiation, anatomical structural development) and protein folding as salinity decreased to 5, indicating an increased need for protein and cell repair. At high Pb levels (3.2 mg/L), GO enrichment at salinity 25 indicated active resistance and attempts to remove Pb toxicity through transmembrane activity and vesicle-mediated expulsion. As salinity levels decreased to 5, these categories were replaced by structural changes in the cell, indicating a need for cell renewal, damage repair, and nitrogen cycle metabolic processes, which may be involved in attempts to reduce toxicity. This was emphasised in the only significant GO enrichment in this experiment, where antioxidant activity was enhanced in AHighS05 vs AControl (Supplementary File S4). When different Pb levels at the same salinity were compared at a salinity of 25, there was enrichment in membrane transport and circulation, thereby reducing the toxic load on cells. At a salinity of 15, there was enrichment of DNA repair processes and metabolism of RNA and proteins. Finally, at the lowest salinity (5), enrichment indicated attempts to increase energy levels and reduce toxicity via vesicle-mediated transport (Supplementary File S3).

Experiment B was performed at 30 °C, and this higher temperature impacted the mussels at all salinities, with enrichment for DNA repair and programmed cell death evident in many of the comparisons, especially at the lower salinity of 15 (Supplementary File S3). In particular, programmed cell death was significantly enriched in HighS15 compared with HS25, indicating increased stress and shutdown of cellular metabolism in the lower-salinity treatment.

3.6. Differential expression of chaperones and "stress" genes

Experiment A, in particular, showed the first line defence of chaperone proteins in the combat against the effects of reduced salinity and Pb addition (Table 2; Supplementary File S4).

Chaperones were present in the cells of animals subjected to both low

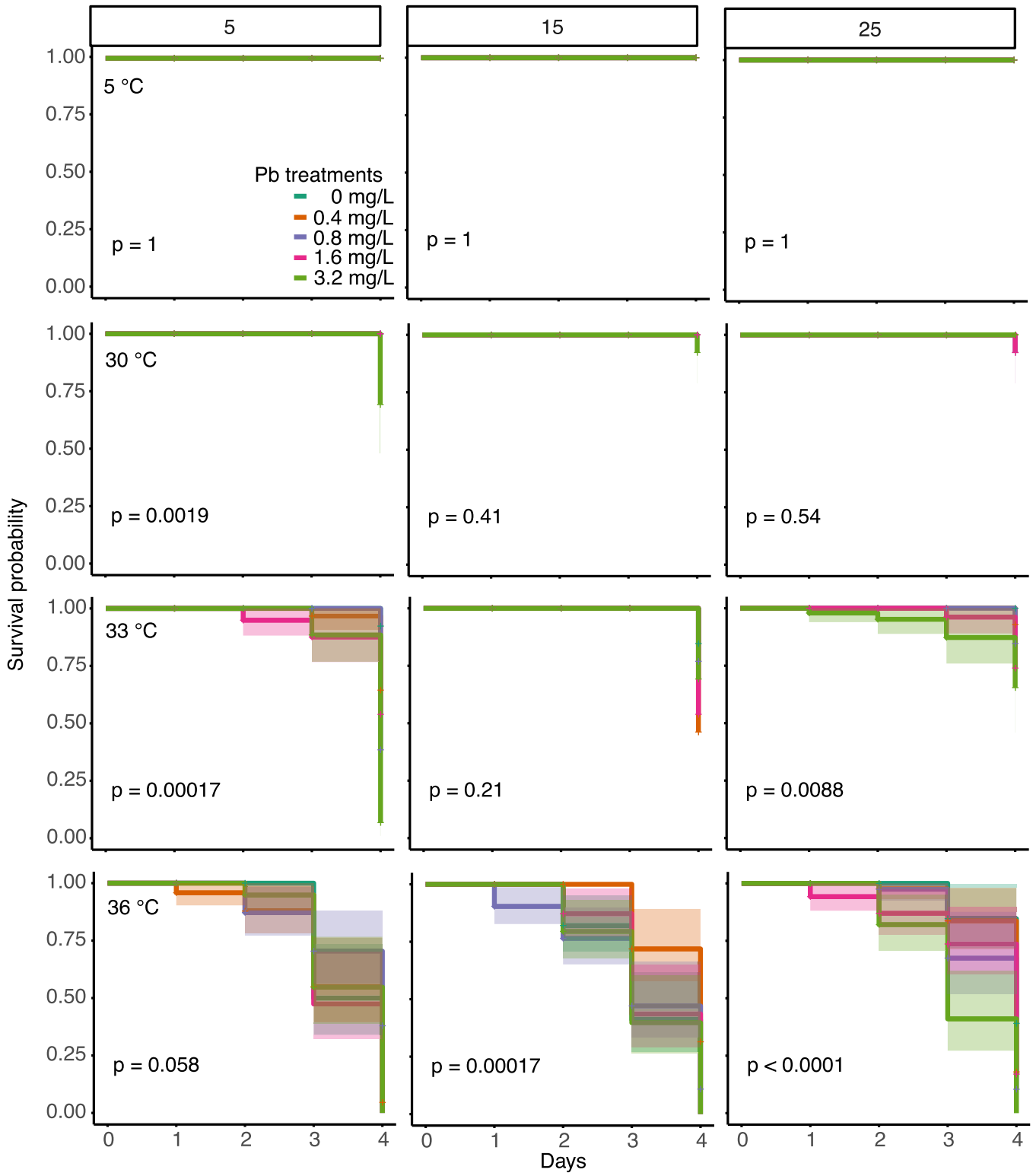

Fig. 3. Kaplan–Meier survival curves of Pb-contaminated *Mytilus edulis* during four consecutive days of repeated aerial exposure across combinations of salinity (columns: 5, 15, and 25) and air temperature (rows: 5, 30, 33, and 36 °C). Colored lines represent the five Pb exposure levels (0, 0.4, 0.8, 1.6, and 3.2 mg Pb/L) applied during a 10-day contamination phase preceding the experiment. Shaded areas indicate 95% confidence intervals. The *p*-values correspond to log-rank tests comparing survival among Pb treatments within each treatment.

and high Pb levels, with the atypical HSP70 genes (HSP7012A and HSP7012B) and BiP (Endoplasmic Reticulum Chaperone) predominating. At high Pb levels, there was an increased presence of classical defence genes such as protein disulphide isomerase, glutathione-S-transferases, and cytochrome P450 in the lower-salinity experiments, indicating a more robust defence against increased toxicity. When different Pb levels at the same salinity were analysed (i.e., AHighS25 vs. ALowS25), there was little difference associated with increased Pb levels beyond the expression of a few immune-related and defence genes, with the latter appearing at the lower salinity comparisons. There were lower

Table 1
Levels of differential expression under different experimental conditions.

Comparison	Differential expression		
	Up	Down	Total
Experiment A			
Low Pb levels (0.8 mg/L), different salinities			
ALowS25vsAControl	401	316	717
ALowS15vsAControl	285	288	573
ALowS05vsAControl	220	215	435
High Pb levels (3.2 mg mg/L), different salinities			
AHighS25vsAControl	472	222	694
AHighS15vsAControl	421	313	734
AHighS05vsAControl	460	303	763
Different Pb levels, same salinities			
AHighS25vsALowS25	153	231	384
AHighS15vsALowS15	217	222	439
AHighS05vsALowS05	209	155	364
Experiment B			
Same Pb level, different salinities			
BControlS15vsBControlS25	87	136	223
BLowS15vsBLowS25	88	163	251
BHighS15vsBHighS25	145	193	338
Salinity 25, different Pb levels			
BLowS25vsBControlS25	253	152	405
BHighS25vsBControlS25	278	257	535
Salinity 15, different Pb levels			
BLowS15vsBControlS15	168	99	267
BHighS15vsBControlS15	151	130	281

DESeq2 results using padj< = 0.05 |log2(Fold Change)|> = 1.0.

levels of "stress" gene differential expression in Experiment B, which may reflect the additive stress of heat, having been conducted at 30 °C. Heat shock proteins were present only in cells at a salinity of 25 at both low and high Pb levels, along with defence genes such as caspase and protein disulphide isomerase. Immune genes predominated at salinity 15, low Pb levels, and genes designated as apoptosis inhibitors appeared at both salinity 15 and 25 at high Pb levels (Table 2; Supplemental File S4).

4. Discussion

Understanding the mechanisms underlying stress resilience is key to predicting the impacts of rapidly changing environmental conditions. In our experiment, lead (Pb) contamination reduced survival in blue mussels (*Mytilus edulis*) after aerial exposure. When the stressors of Pb, salinity, and temperature were applied sequentially, blue mussel survival exhibited complex interaction patterns consistent with partial cross-tolerance. Specifically, the survival analyses suggest that mussels initially exposed to higher Pb concentrations displayed significantly greater resilience to warming, whereas decreasing salinities increased sensitivity to heat. At the molecular level, this was evidenced by an increased expression of classical stress response genes, including protein disulphide isomerase, glutathione-S-transferases, cytochrome P450, and chaperones in the higher Pb-treated animals (Table 2; Supplementary File S5). These are transcripts previously identified in the transcriptomic responses of *Magallana gigas* (previously *Crassostrea gigas*) and marine copepods to a range of xenobiotics, and all are well-known components of the cellular stress response (CSR) (Kültz, 2005; Lüchmann et al., 2015). Furthermore, the prominence of the atypical HSP70 family, HSP70A12 and HSP7012B, among the up-regulated chaperones across all treatments (Supplementary File S5) further validates their roles as important elements of the CSR and as intertidal stress sensors (Clark et al., 2021). Importantly, the transcriptional activation of chaperones and detoxification pathways occurred under the same treatment conditions that attenuated temperature-driven mortality, providing mechanistic coherence between organismal survival patterns and cellular stress responses.

Exposure to Pb-contaminated seawater resulted in net Pb uptake in soft tissues, but the uptake decreased in mussels exposed to the highest Pb treatment (3.2 mg/L). This decline is unlikely to be attributed to active excretion because Pb is only slowly excreted from mussels (Regoli and Orlando, 1994). Instead, individuals exposed to 3.2 mg Pb/L seawater may have reduced their filtration rates or valve opening to minimize exposure to the highly polluted water. Still, we successfully obtained a range of environmentally realistic Pb tissue levels, with the highest average (1481.25 μg/g dw) close to Pb pollution levels (~1500 μg/g dw) in blue mussels at decommissioned mining sites in Greenland (Søndergaard et al., 2010). While we show that Pb contamination increases mortality in mussels exposed to air temperatures above 30 °C, previous work showed that exposure to subzero air temperatures had no effect on survival in Pb-contaminated blue mussels (Thyrring et al.,

Table 2
Identification of genes related to the stress response in the different treatments.

Experiment	No of up-regulated genes	up-regulated genes with SwissProt annotation	No of "stress" genes identified	Category of "stress" gene			
				Chaperones	Defence	Immune	Apoptosis
Experiment A							
Low Pb levels (0.8 mg/L), different salinities							
ALowS25vsAControl	401	131	14 (11%)	11	1	1	1
ALowS15vsAControl	285	63	21 (33%)	10	4	7	0
ALowS05vsAControl	220	36	10 (28%)	8	0	1	1
High Pb levels (3.2 mg/L), different salinities							
AHighS25vsAControl	472	57	13 (23%)	7	5	1	0
AHighS15vsAControl	421	114	36 (32%)	11	13	11	1
AHighS05vsAControl	460	113	33 (29%)	13	16	4	0
Different Pb levels at same salinities							
AHighS25vsALowS25	153	12	3 (25%)	0	0	3	0
AHighS15vsALowS15	217	27	5 (19%)	1	0	3	1
AHighS05vsALowS05	209	23	5 (22%)	1	2	2	0
Experiment B							
Same Pb level, different salinities							
BControlS15vsBControlS25	87	12	0 (0%)	0	0	0	0
BLowS15vsBLowS25	88	11	1 (9%)	0	0	1	0
BHighS15vsBHighS25	145	19	2 (11%)	0	1	1	0
Salinity 25, different Pb levels							
BLowS25vsBControlS25	253	33	4 (12%)	2	1	1	0
BHighS25vsBControlS25	278	36	10 (28%)	6	3	1	0
Salinity 15, different Pb levels							
BLowS15vsBControlS15	168	19	6 (32%)	0	2	4	0
BHighS15vsBControlS15	151	14	3 (14%)	0	2	1	0

2015). However, the physiological processes behind surviving heat and freezing vary and require distinct specialized molecular responses. Valve gaping and the expression of chaperone proteins, such as heat shock proteins (HSPs) (Clegg et al., 1998), are common mechanisms for surviving high-temperature exposure, while freeze tolerance mechanisms used by marine invertebrates include the accumulation of cryoprotectants or antifreeze proteins (Kennedy et al., 2020). Because Arctic intertidal species experience both high summer temperatures and sub-zero winter conditions (Thyrring et al., 2019), future research should examine how pollution affects both upper and lower thermal tolerance limits to understand the ecological implications.

Intertidal air temperatures in Greenland can exceed 36 °C (Thyrring et al., 2017), and repeated exposure to high air temperatures results in cumulative mortality (Nielsen et al., 2021). Exposure to additional stressors can result in additive, synergistic, or antagonistic effects. We show a significant negative interaction between Pb and air temperature, which indicates that the temperature-related increase in mortality risk is attenuated at higher Pb concentrations during repeated aerial exposure. Thus, although Pb contamination increased baseline mortality, its presence reduced the incremental effect of high air temperatures, suggesting an antagonistic interaction between metal contamination and thermal stress. Under repeated high air temperature exposure, the relative impact of warming was therefore weaker in Pb-contaminated mussels than in uncontaminated individuals. Such an antagonistic effect is consistent with cross-tolerance (Collins et al., 2023), whereby prior Pb exposure induces physiological pathways that moderate subsequent heat-induced mortality. Specifically, the primary application of the Pb treatment with up-regulation of stress response genes provides cross-tolerance to subsequent stressors through a process often called "preparative defense" and "transcriptional (constitutive) frontloading" (Barshis et al., 2013; Dong et al., 2008). Such effects have been demonstrated across a range of species, from limpets to crustacea and fish, particularly in the intertidal and tidal pools (Clark et al., 2018; Collins et al., 2021; Denny and Dowd, 2022; Todgham et al., 2005). In our study, the attenuation of temperature-driven mortality at higher Pb exposure coincided with the up-regulation of chaperones and detoxification-related transcripts, supporting the hypothesis that activation of the cellular stress response contributed to the observed survival patterns. Thus, pollution can modify thermal sensitivity in non-additive ways, complicating predictions of organismal responses to Arctic climate warming. Given the increasing frequency of Arctic marine heatwaves and continued legacy metal inputs from historical mining areas, such multi-stressor interactions are likely to become more common in Greenlandic fjord systems.

The majority (over 90%) of the BLAST matches in the RNA-seq experiments were against other *Mytilus* species, such as *M. galloprovincialis* (33.5% and 36.1% of the total for Experiments A and B, respectively), *M. edulis* (29.1% and 31.3%), *M. coruscus* (15.6% and 16.1%) and *M. californianus* (16.6% and 12.9%). BUSCO assessment for both experiments was greater than 90% complete. The molecular response was overall relatively muted, showing, at most, differential expression of a few hundred genes across treatments. Whilst initially surprising, on reflection, these results fit well with current knowledge of mussels from the *Mytilus edulis* species complex, which are stress-resilient and successful global colonisers (Wenne et al., 2020). *Mytilus* species can successfully survive in a wide range of different environmental conditions, including the Baltic, where salinities can be as low as ~5 (Kautsky, 1982). A particular adaptation in this example is thought to be due to this species' ability to use a significant amount of meat biomass (up to 78%) as an energy reserve under adverse conditions (Kautsky, 1982). *Mytilus edulis* from the Nuuk area in Greenland (as used in this project) have previously been the subject of molecular analyses to study their stress responses to changing environmental conditions (Barrett et al., 2022; Clark et al., 2021). These studies have highlighted different responses to natural and experimentally induced stressors, as well as the experimental use of dual stressors (temperature and salinity) (Barrett et al., 2022; Clark et al., 2021), demonstrating the highly plastic nature of this species. For instance, intertidal populations in Greenland can experience temperature differences of over 20 °C during a low-tide cycle (depending on location), and yet when examined via RNA-Seq, these populations showed no significant differences in functional gene expression (Clark et al., 2021). Such plasticity is thought to result from adaptation to living in a highly fluctuating yet predictable environment (Drake et al., 2017; Wang et al., 2020). This was in stark contrast to experimental temperature manipulation, which was conducted simultaneously on the same populations. An acute thermal challenge resulted in markedly different, more pronounced changes in gene expression levels and in altered functionality of expressed products (Clark et al., 2021). A subsequent experiment examining the response of *M. edulis* to both temperature and salinity highlighted the fact that, as expected, the application of dual stressors reduced the species' tipping point (Barrett et al., 2022). However, there was substantial overlap among genes responsible for salinity and heat tolerance, indicating stimulation of similar components of the CSR. This suggested that the sequential application of salinity and heat stress (as opposed to simultaneous application in the published study) could lead to cross-tolerance and enhanced protection for *M. edulis* in a multi-stressor world (Barrett et al., 2022). In our experiment, the attenuation of temperature-driven mortality at higher Pb exposure coincided with the up-regulation of chaperones and detoxification-related transcripts, supporting the hypothesis that activation of the cellular stress response contributed to the observed survival patterns. Thus, our study on the effects of three different stressors expands knowledge and enhances understanding of this topic. However, it should be noted that physiological responses are complex and depend on the temporal dynamics of the multiple stressors, namely intensity, timing, and order (Rodgers and Gomez Isaza, 2023). For example, in our study, there was a significant interaction between Pb and salinity, with the protective effect of salinity against thermal stress decreasing as Pb content increased. In our experiments, Pb was the first stressor introduced and therefore appeared to have the greatest protective effect compared with the additional salinity and temperature treatments. Whether there would have been a greater protective effect if the stressors had been applied in a different order or on different temporal scales cannot be determined here, but it is clearly an important question to ask in future studies.

Ultimately, the application of an increasing number of stressors to any species results in lower tipping points and the activation of apoptosis pathways, as demonstrated here (Table 2; Supplementary Table S5). Our results demonstrate that *M. edulis* exposed to different Pb concentrations induces preparatory activation of the cellular stress response, which may facilitate cross-tolerance to additional stressors, revealing the potential for short-term resilience in future oceans. Indeed, there is growing evidence that cross-tolerance is induced by emerging pollutants such as pesticides and pharma (Bridge et al., 2024; Rodgers et al., 2025). Our findings indicate that legacy metal contamination may reshape thermal-vulnerability landscapes in Arctic intertidal ecosystems under accelerating climate change, but the long-term population consequences remain uncertain.

CRediT authorship contribution statement

Melody S. Clark: Writing – review & editing, Visualization, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Jakob Thyrring:** Writing – original draft, Visualization, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Rosa Freitas:** Writing – review & editing, Methodology, Funding acquisition, Formal analysis.

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Declaration of Competing Interest

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2026.120601.

Data availability

Raw experimental data supporting the findings of this study are openly available in Zenodo at https://doi.org/10.5281/zenodo.21715583 (Thyrring et al., 2026). The RNA-seq data have been deposited in the EBI ArrayExpress database with the accession number E-MTAB−16721 (https://www.ebi.ac.uk/biostudies/arrayexpress).

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