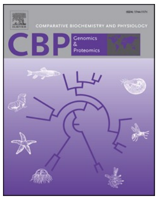
Comparative Biochemistry and Physiology - Part D: Genomics and Proteomics 58 (2026) 101778

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology - Part D: Genomics and Proteomics

journal homepage: www.elsevier.com/locate/cbpd



# Population-specific phenotypic plasticity of endangered bivalves in response to extreme events

André Gomes-dos-Santos[a,*], Manuel Lopes-Lima[b,c], Beatriz Silva[a], André M. Machado[a], Rui Pinto[a], Martin Österling[d], Magnus Lovén Wallerius[e], Maria Urbańska[f], Janusz Golski[f], Sławomir Runowski[f], Sandra Kaźmierczak[f], Amílcar Teixeira[g], Ronaldo Sousa[h], L. Filipe C. Castro[a,i], Paulo Castro[h], Francisco Carvalho[h], Elza Fonseca[a], Elsa Froufe[a,j,*]

[a] *Aquatic Ecology and Evolution (AEE) group - Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Terminal de Cruzeiros do Porto de Leixões, 4450-208, Matosinhos, Portugal*
[b] *CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório; Associado, Universidade do Porto, Vairão, Portugal*
[c] *BIOPOLIS Program in Genomics, Biodiversity and Land Planning, CIBIO, Vairão, Portugal*
[d] *River Ecology and Management, Research Group RivEM, Department of Environmental and Life Sciences, Karlstad University, Karlstad, Sweden*
[e] *Department of Biological and Environmental Sciences, University of Gothenburg, Gothenburg, Sweden*
[f] *Department of Zoology, Poznań University of Life Sciences, ul. Wojska Polskiego 28, 60-637, Poznań, Poland*
[g] *CIMO, LA SusTEC, Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253, Bragança, Portugal*
[h] *CBMA – Centre for Molecular and Environmental Biology/ARNET-Aquatic Research Network & IB-S, Institute of Science and Innovation for Bio-Sustainability, Department of Biology, University of Minho, 8 Campus Gualtar, 4710-057, Braga, Portugal*
[i] *Department of Biology, Faculty of Sciences University of Porto, Portugal*
[j] *ICBAS - Laboratory of Cytogenetics, School of Medicine and Biomedical Sciences, University of Porto, Portugal*

## ARTICLE INFO

Edited by Chris Martyniuk

*Keywords:*
Phenotypic plasticity
Differential gene expression (DGE)
Climate change
Heat stress
Unionida

## ABSTRACT

Freshwater mussels are among the most endangered animal groups, highly sensitive to climate change due to their strict dependence on freshwater habitats. While freshwater mussels are often considered ecologically strict, their distribution across broad environmental gradients raises the possibility of population-specific adaptations mediated by phenotypic plasticity.

This study investigates whether geographically and climatically distinct populations of two freshwater mussel species (*Unio pictorum* and *Unio delphinus*) exhibit different transcriptomic responses to prolonged heat stress and whether these responses reveal signs of local adaptation.

We exposed northern and southern populations of both species to gradually increasing temperatures in controlled laboratory conditions, simulating a prolonged thermal extreme event, and RNA-seq was used to quantify differential gene expression.

Results showed strong differences between northern and southern populations of the two species, both in the magnitude and functional composition of transcriptomic responses. Southern populations exhibited intense expression shifts involving classical stress pathways, heat shock proteins, detoxification (cytochrome P450s), apoptosis, and energy metabolism, while northern populations, particularly *U. delphinus*, showed a markedly subdued response. Notably, *U. pictorum*'s northern population relied heavily on the cytochrome P450 family even at moderate temperatures, while the southern populations of both species activated broader proteostasis and immune responses at higher stress thresholds.

These findings demonstrate clear population-specific phenotypic plasticity, shaped by environmental conditions rather than phylogenetic proximity. They underscore the need for conservation strategies to move beyond species-level management, embracing intraspecific variation as a buffer against climate impacts. As climate change accelerates, safeguarding the evolutionary potential encoded within populations, not just species, is essential to preserving biodiversity resilience.

---

[*] Corresponding authors at: Aquatic Ecology and Evolution (AEE) group - Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Matosinhos, Portugal.

*E-mail addresses:* asantos@ciimar.up.pt (A. Gomes-dos-Santos), efroufe@ciimar.up.pt (E. Froufe).
@AndreMaGoSaP (A. Gomes-dos-Santos)

https://doi.org/10.1016/j.cbd.2026.101778
Received 14 November 2025; Received in revised form 28 January 2026; Accepted 6 February 2026

## 1. Introduction

Freshwater ecosystems are a global concern in biodiversity decline (Markovic et al., 2017; Wacker et al., 2024; Sayer et al., 2025). These ecosystems support 10% of all known species, of which 25% are threatened with extinction (Sayer et al., 2025). Climate change stands out as a major concern, imposing an unpredictable and escalating pressure through shifts in the frequency and magnitude of thermal events (Wacker et al., 2024). As a result of climate change, global temperatures are likely to surpass 1.5–2 °C above pre-industrial levels by the mid-21st century (1850–1900) (IPCC, 2023; Capon et al., 2021; Markovic et al., 2017). This extends to aquatic ecosystems, where even small temperature increases may threaten biodiversity (Capon et al., 2021). This is especially critical for sessile freshwater organisms, such as bivalves, which are unlikely to shift their distribution and for whom phenotypic plasticity (metabolic, physiological, and behavioural) constitutes the primary means of response (Luo et al., 2014; Le Luyer et al., 2022; Cushway et al., 2025).

Freshwater mussels (Order Unionida) are a highly diverse group of bivalves distributed globally (Graf and Cummings, 2007; Graf and Cummings, 2022; Graf and Cummings, 2006) and are receiving global attention as one of the most endangered animal groups (Lopes-Lima et al., 2021, 2018; Sousa et al., 2023; Aldridge et al., 2022). Mass mortalities, within species of the group, have been associated with heatwaves, and several species are believed to live near their upper thermal tolerance (Ganser et al., 2015; Gough et al., 2012; McDowell and Sousa, 2019; Nogueira et al., 2021; Sousa, 2018; Cushway et al., 2025). Notably, many species are distributed across a wide range of environments, with populations exposed to varying thermal regimes (Luo et al., 2014; Wang et al., 2015; da Silva, 2022, 2023, 2024), suggesting high potential for locally acquired phenotypic plasticity.

Given their Triassic origin, freshwater mussels have persisted through multiple global climatic fluctuations (Graf and Cummings, 2007; Graf and Cummings, 2006). The current distribution and genetic diversity of many species still bear strong signatures of recent Quaternary climate changes (Froufe et al., 2014, 2016; Keogh et al., 2025). Evolutionarily, these environmental changes likely promoted adaptive mechanisms, shaped by both genetic diversity and an underlying phenotypic plasticity (Le Luyer et al., 2022). Phenotypic plasticity, as a rapid-response mechanism, may be critical for enabling species to withstand the current pace of environmental change, acting as a buffer until longer-term genetic adaptation can occur (Le Luyer et al., 2022). Consequently, understanding how distinct populations of the same species, adapted to contrasting environments, respond to abrupt temperature increases will provide valuable insights into the true impact of climate change. Phenotypic plasticity may arise through several genetic mechanisms (Scheiner, 1993; Ding et al., 2012; Gerdol et al., 2020). Gene expression variability, modulated by a complex interplay of genetic and epigenetic modifications, is often the primary driver of phenotypic responses (Duncan et al., 2014; Ghalambor et al., 2015). Transcriptomic approaches frequently rely on comparisons of gene expression profiles under different environmental conditions. This makes them a powerful tool for investigating phenotypic plasticity and physiological responses (Kelly, 2019; Rivera et al., 2021).

In this study, we used transcriptomic data to investigate how ecologically and geographically distinct populations of two freshwater mussel species respond to a simulated prolonged thermal extreme event. The focal species are *Unio pictorum* (Linnaeus, 1758), widely distributed across Central and northern Europe, and *Unio delphinus* Spengler, 1793, an Iberian endemic. These species exhibit considerable habitat plasticity, occupying diverse freshwater ecosystems across different ecoregions (Lopes-Lima et al., 2017), making them ideal models for exploring local adaptive responses. Individuals from two geographically separated populations of each species were exposed to a gradually increasing temperature regime under controlled laboratory conditions, and gill tissues were used for RNA-seq-based gene expression analyses.

Our findings reveal pronounced population-specific responses to thermal stress within and between species, indicating the potential existence of local phenotypic plasticity influenced by environmental history. These results emphasise the importance of considering intraspecific variation in phenotypic plasticity when evaluating the effect of climate change and developing conservation strategies for freshwater mussels.

## 2. Materials and methods

### 2.1. *Mussel sampling and laboratory acclimation*

Adult individuals were collected in the summer of 2023. Specimens of *Unio pictorum* were collected from Viskan River in Sweden (57.759944, 12.941361, water temperature 20 °C) ($n = 9$) and Pilica River in Poland (50.909886, 19.801080, water temperature 20 °C) ($n = 9$). Specimens of *Unio delphinus* were collected from the Rabaçal River in northern Portugal (41.409246, −7.165456, water temperature 26 °C)($n = 15$) and Vidigão River in southern Portugal (37.892583, −7.303778, water temperature 27 °C)($n = 15$). All mussels were transported to the laboratory and acclimated at 15 °C for two weeks in individual tanks containing air-oxygenated freshwater (20 cm × 30 cm). During acclimation, individuals were fed daily with a microalgae solution.

### 2.2. *Laboratory experiments*

Three distinct temperatures were set as targeted test conditions: 20 °*C*, 25 °*C*, and 30 °*C*. For each species, populations and target conditions, collected individuals were divided evenly in individual tanks, i.e., 3 *U. pictorum* individuals per tank (total of 6 tanks and 18 individuals) and 5 *U. delphinus* individuals per tank (total of 6 tanks and 30 individuals) (Fig. 1). The individuals started at the acclimation temperature of 15 °C, subsequently, the temperature was gradually increased ~1 °C /d until it reached three distinct temperatures: 20 °*C*, 25 °*C*, and 30 °*C*. After reaching each of the target temperatures, the individuals were held for two weeks. Due to a technical malfunction, the tank housing the southern population of *U. pictorum* intended for exposure to 20 °C remained at the acclimation temperature of 15 °C. Despite this, samples were retained as they still provide meaningful data for within-population comparisons. The lower temperature of each replicate was considered the control in gene expression analyses. At the end of each experimental condition, gill samples were collected and preserved in *RNAlater* Stabilisation Solution at 4 °C for 24 h and subsequently at −80 °C at CIIMAR, Portugal. Gill was selected for transcriptomic analysis, in line with standard practice in stress studies on freshwater mussels and other bivalves (Li et al., 2017; Robertson et al., 2017; Roznere et al., 2021; Beninger et al., 1993; Jørgensen, 1974; Pourmozaffar, 2020; Tankersley, 1996).

### 2.3. *RNA extraction, library construction, and NGS sequencing*

Total RNA was extracted from the gill samples of all individuals ($n = 48$) using the NZYTech total RNA isolation kit and an on-column DNase I treatment. Extracted RNA was sent to Macrogen, Inc. to build strand-specific libraries (250–300 bp insert size) and paired-end (150 bp) sequencing using an Illumina HiSeq 4000 platform.

### 2.4. *RNA-Seq analysis*

#### 2.4.1. *Pre-processing*

The quality of RNA-Seq data was assessed using FastQC (version 0.11.8, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) with default parameters. Trimmomatic (version 0.38) was used to quality-filter and remove Illumina adapters, with the parameters LEADING:5 TRAILING:5 SLIDINGWINDOW:5:20 MINLEN:36 (Fig. 1).

### 2.4.2. *Genome alignment and expression quantification*

The reference genome (and respective annotation) of *U. pictorum* and *U. delphinus* were retrieved from public databases (Gomes-dos-Santos et al., 2023a, 2023b), and used as references for RNA-seq read alignment (Fig. 1). The annotations files were converted into a GTF using AGAT (version 1.0.0), with the script agat_convert_sp_gxf2gxf.pl and indexed using HISAT2 hisat2-build (version 2.2.1). The clean reads were mapped to the respective genomes using the software HISAT2 (version 2.2.1) with the default parameters. Following the RNA-Seq read alignment, the data were quantified using Stringtie (version 2.1.2) with the parameters -e —B -p 30 -G and prepDE.py to get the read counts per gene.

### 2.4.3. *Differential gene expression (DGE)*

Differential gene expression (DGE) was performed with Degust (http://degust.erc.monash.edu/) (Fig. 1). The read counts table was imported to Degust, and all genes with less than one count per million mapped reads in at least three samples of the same condition for *U. pictorum* and five samples for *U. delphinus* were excluded. Differential Gene Expression estimations were conducted with edgeR (v.4.2.1) R package (Robinson et al., 2010) (R v.4.4.1) applying the normalisation scale with the trimmed mean of M-values (TMM) method (Robinson and Oshlack, 2010). Variance between samples and experiments was tested using a multidimensional scaling (MDS) plot, using solely the filtered and normalised data. Detection of differentially expressed genes was tested for each experiment, with the lowest temperature exposure established as the control (Table 1), in which only genes with a corrected False Discovery Rate (FDR) *p*-value $<0.05$ and log2|fold change| $\geq 2$ were considered differentially expressed. Heatmaps for the inferred DEGs were obtained using the Heatmapper (Babicki et al., 2016) with the clustering method (Average Linkage) and the Distance measurement method (Pearson) applied to the rows of the dendrogram.

### 2.4.4. *Gene annotation mining and enrichment analysis*

Using the genome's functional annotations, differently expressed

**Table 1**
Number of differentially expressed genes between each condition for intra-population comparison.

| | | North | | South | |
|---|---|---|---|---|---|
| *Unio pictorum* | Experiment | 20°C ∩ 25°C | 20°C ∩ 30 °C | 15 °C ∩ 25 °C | 15 °C ∩ 30 °C |
| | Total DGE | 33 | 127 | 154 | 4029 |
| | FC relative to | North 20 °C | North 20 °C | South 15 °C | South 15 °C |
| Log2 Fold-change | Down-regulated | 6 | 81 | 117 | 2253 |
| | Up-regulated | 27 | 46 | 37 | 1776 |
| *Unio delphinus* | Experiment | 20°C ∩ 25°C | 20°C ∩ 30 °C | 20°C ∩ 25 °C | 20°C ∩ 30 °C |
| | Total DGE | 1 | 2 | 7 | 1803 |
| | FC relative to | North 20°C | North 20 °C | South 20 °C | South 20 °C |
| Log2 Fold-change | Down-regulated | 0 | 2 | 3 | 788 |
| | Up-regulated | 1 | 0 | 4 | 1015 |

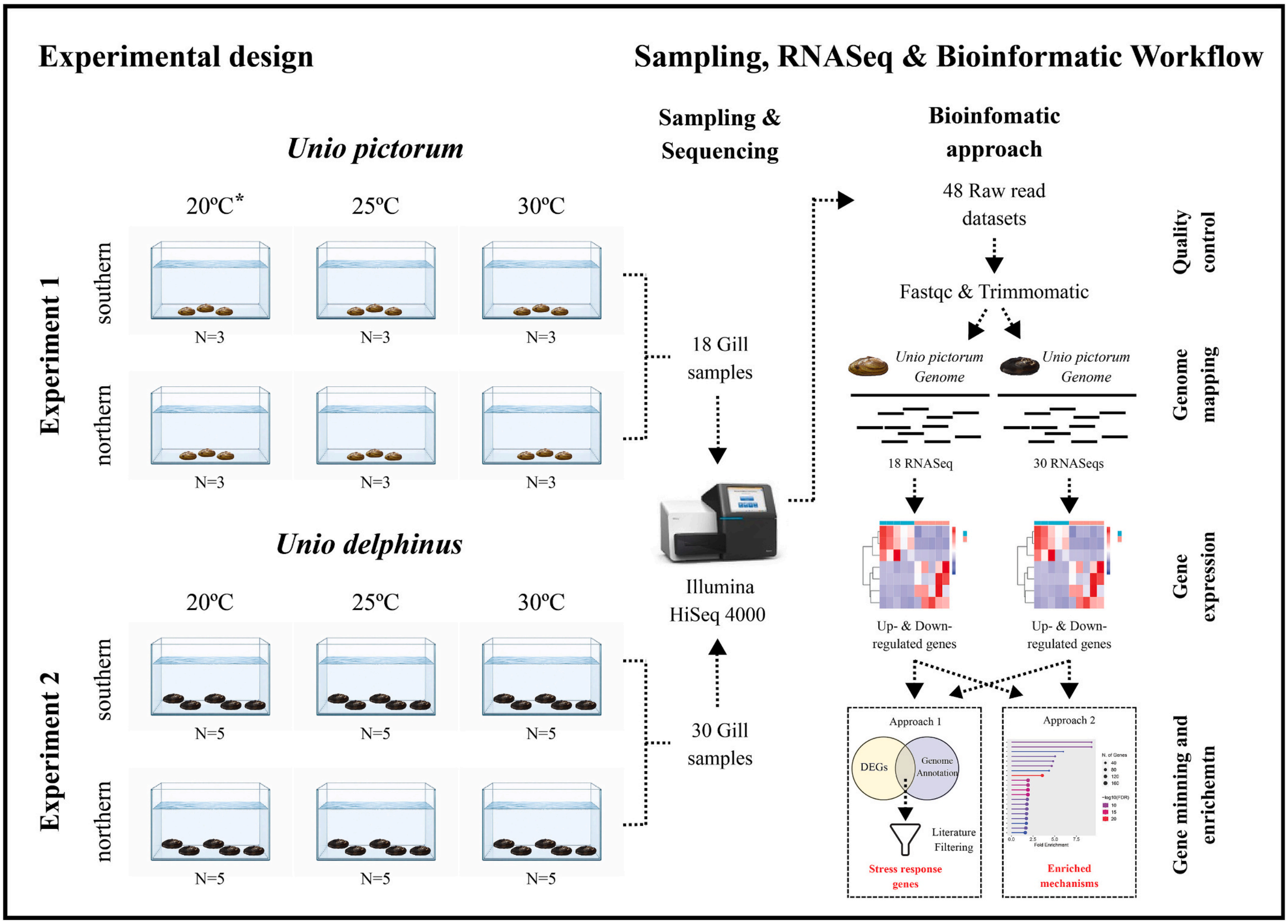


**Fig. 1.** Graphical schematization of the heat stress ecological experimental design (left) and the differential gene expression bioinformatic pipeline (right). * See text for details.

genes were mined using “word cues” of genes and gene families known to be related to stress response in freshwater mussels and other bivalves (Supplementary File 1) (Fig. 1).

Complementary, an enrichment analysis was performed using ShinyGO (v.0.82) (Ge et al., 2020). Briefly, a BLAST homology search of the proteins coding DEGs was conducted against the proteome of *Homo sapiens* (downloaded from Ensembl - GRCh38), using the blastp from BLAST+ (version 2.14.0) (Camacho et al., 2009), specifying parameters -max_target_seqs 1 -max_hsps 1 -evalue 1e-5. The resulting gene ENSEMBL accessions were supplied to ShinyGO (v.0.82) (Ge et al., 2020) online tool (http://bioinformatics.sdstate.edu/go/) (Ge et al., 2020) and use to search for enrichment in the GO terms databases, Biological Process (BP), Molecular Functions (MF) and Cellular Components (CC), utilising a false discovery rate (FDR) cutoff with $\rho < 0.05$.

## 3. Results

### 3.1. *Genome mapping, differential gene expression (DGEs) analyses and functional characterisation*

The percentage of read alignment to the reference genomes was above 90% for all samples. All the differential gene expression analyses were conducted starting with a total of 41,435 genes for *U. pictorum* and 39,359 genes for *U. delphinus* (Supplementary File 2). To characterise differently expressed genes in response to gradual temperature increases, individuals reared at 25 °C and 30 °C were compared to the lower temperature group (i.e., 20 °C, or 15 °C in the southern population from Poland).

Two types of strategies were used for functional characterisation: 1) filtering the differentially expressed gene tables using a list of names of stress-related gene families and genes (Supplementary File 1); and 2) enrichment analyses based on Gene Ontology (Fig. 1).

#### 3.1.1. *Unio pictorum northern population - Sweden*

The analyses revealed a low number of differentially expressed genes (DEGs), showing an increase in DEGs as the temperature increased. Individuals at 20 °C–25 °C revealed 33 DEGs, while 20 °C–30 °C revealed 127 DGEs (Table 1). Additionally, opposite patterns were found for up and down gene expression for each condition. While most genes in the 20 °C–25 °C condition were up-regulated (27 up, six down), most genes in the 20 °C–30 °C condition were down-regulated (46 up, 81 down) (Table 1; Fig. 2). Importantly, both conditions shared 12 DEGs that exhibited constitutive expression as the temperature increased (Fig. 2c).

To highlight the biological functions of the DEGs we applied two different approaches. Firstly, we gathered functional annotations for DEGs from the reference genome. Next, we filtered the annotated DEGs using a selection of “word cues” for genes and gene families known to be related to the stress responses (Supplementary File 1). Secondly, we

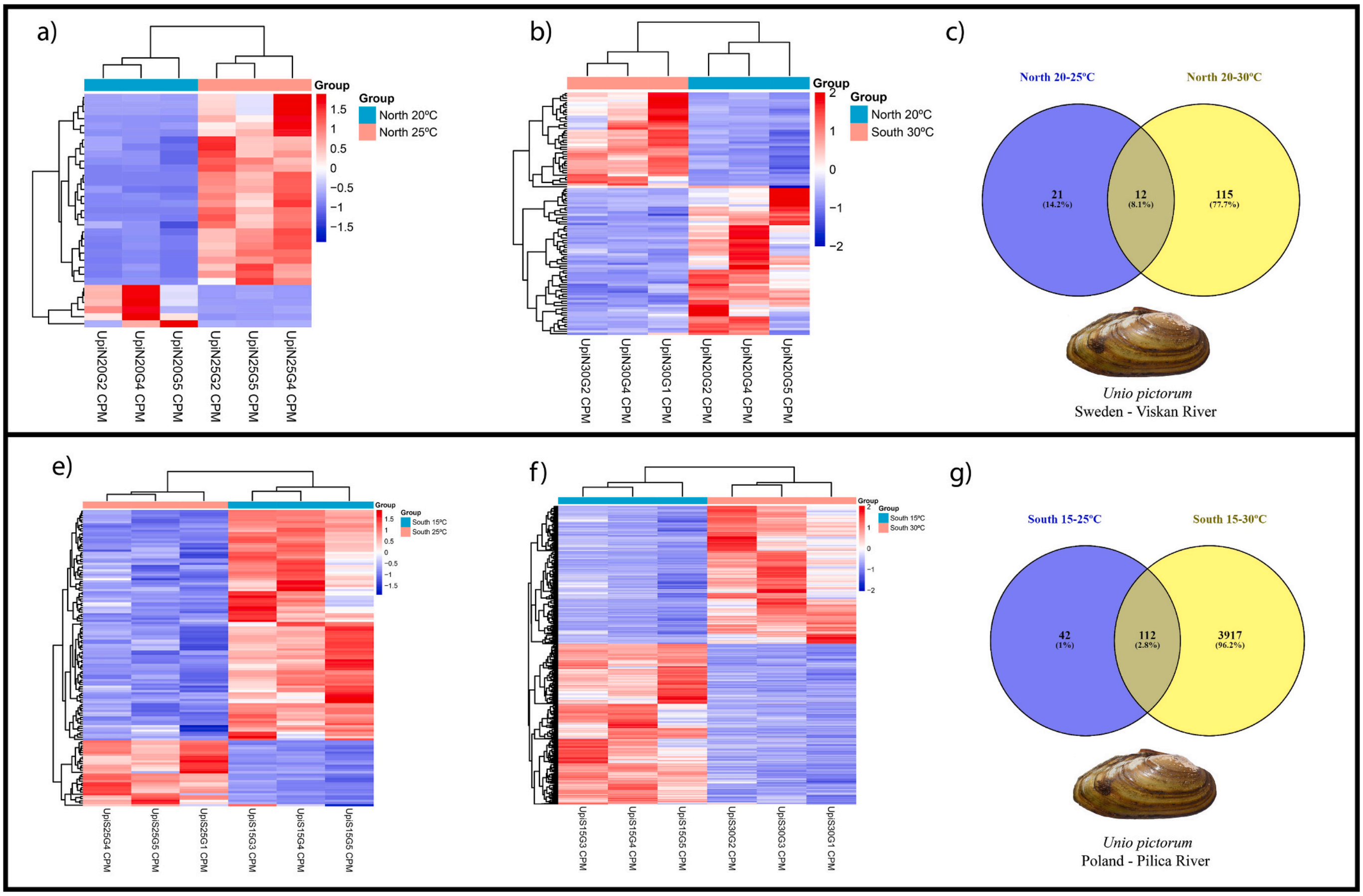


**Fig. 2.** Results of differentially expressed genes analyses among individuals of *Unio pictorum* cultured under different temperature conditions. a-c) Northern population (n = 9), from Sweden. a) Hierarchical Heatmap corresponding to individuals reared at 25 °C compared to the control temperature of 20 °C (North 20 °C ∩ North 25 °C). b) Hierarchical Heatmap corresponding to individuals reared at 30 °C compared to the control temperature of 20 °C (North 20 °C ∩ North 30 °C). c) Venn diagram of reporting the intersection of differentially expressed genes between the two tested conditions. e-g) Southern population (*n* = 9), from Poland. e-g) Southern population, from Poland. e) Heatmap corresponding to individuals reared at 25 °C compared to the control temperature of 15 °C (South 15 °C ∩ South 25 °C). f) Heatmap corresponding to individuals reared at 30 °C compared to the control temperature of 15 °C (South 15 °C ∩ South 30 °C). g) Venn diagram of reporting the intersection of differentially expressed genes between the two tested conditions. All heatmaps were constructed using the SRPLOT online tool, using the samples' CPM estimations resulting from Degust.

performed gene ontology enrichment analyses on the annotated and normalised DEGs against the *Homo sapiens* proteome.

Using the first approach, we achieved a significant reduction in the number of genes (see Table 2). Here, we identified 16 out of 33 DEGs and 17 out of 127 DEGs, annotated and correlated with stress responses in conditions 20 °C–25 °C and 20 °C–30 °C, respectively (see Supplementary Table 1). Notably, the majority of this reduction occurred in the initial sub-step of the approach (annotation gathering), where most of the DEGs were annotated as hypothetical or uncharacterised proteins (47 and six in the first and second conditions, respectively.

Among the 16 DEGs identified in 20–25 °C condition, nine were cytochrome P450 genes (eight up-regulated and one down-regulated) (Supplementary Table 1). In the 20–30 °C condition, five cytochrome P450 genes were found to be differentially expressed: two were up-regulated, and three were down-regulated. Importantly, we were still able to identify several other stress-related genes in this condition (e.g., HSP90 co-chaperone CDC37 and ubiquitin-conjugating enzyme E2–34kDa-like).

When we compared the DEGs in both conditions, we found that several genes remained up -or down regulated in both 20–25 °C and 25–30 °C conditions. These include cyclophilin (UpiG00000032055) and a cytochrome P450 (UpiG00000043919), which were both down-regulated, as well as two cytochrome P450s (UpiG00000024934 and UpiG00000020252), both up-regulated at 25 °C and 30 °C (Supplementary Table 1).

The second approach, the enrichment analyses, agreed partially with these results (Supplementary Fig. 2 and Supplementary Table 2). Across the three main Gene Ontology categories ("Biological Processes (BP)", "Cellular Components (CC)" and "Molecular Functions (MF)"), we found the up-regulated genes of the 20–25 °C condition globally linked to metabolism, detoxification, and immune-related functions (see Supplementary Fig. 2 and Supplementary Table 2). Conversely, enrichment analyses could not be performed on the down-regulated genes of 20–25 °C, as the initial blast against *H. sapiens* proteome did not report any hits.

The enrichment analysis results for individuals reared at 20–30 °C also follow a similar pattern (Supplementary Fig. 3; Supplementary Table 3). For up-regulated genes at 20–30 °C, for all GO categories, only one gene ontology term was enriched with an FDR $p < 0.05$, for all GO categories, i.e., "nucleic acid binding" in MF (Supplementary Table 3). Differently, in downregulated genes the enrichment results showed global links to developmental pathways, extracellular matrix regulation, and metabolic and transport functions (Supplementary Fig. 3, Supplementary Table 3).

**Table 2**
Number of differentially expressed genes between each condition for intra-population comparison after filtering gene expression tables by gene names linked to stress response.

| | | North | | South | |
|---|---|---|---|---|---|
| *Unio pictorum* | Experiment | 20°C ∩ 25°C | 20°C ∩ 30°C | 15 °C ∩ 25 °C | 15 °C ∩ 30 °C |
| | Total DGE | 16 | 17 | 27 | 541 |
| | FC relative to | North 20 °C | North 20 °C | South 15 °C | South 15 °C |
| Log2 Fold-change | Down-regulated | 2 | 9 | 21 | 279 |
| | Up-regulated | 14 | 8 | 6 | 262 |
| *Unio delphinus* | Experiment | 20°C ∩ 25°C | 20°C ∩ 30°C | 20°C ∩ 25 °C | 20°C ∩ 30 °C |
| | Total DGE | 1 | 0 | 1 | 237 |
| | FC relative to | North 20°C | North 20 °C | South 20 °C | South 20 °C |
| Log2 Fold-change | Down-regulated | 0 | 0 | 0 | 87 |
| | Up-regulated | 1 | 0 | 1 | 150 |

### 3.1.2. *Unio pictorum southern population - Poland*

In contrast to the experiment on the northern population, a large number of differentially expressed genes were identified in the southern population (see Table 1). Similarly, to the northern experiment, the number of DEGs increased with temperature. In this population, 20–25 °C showed 127 DEGs, while 20–30 °C revealed approximately 4029 DEGs (see Table 1). The distribution of up- and down-regulated genes shows a predominance of down-regulation in both conditions: 81 down-regulated vs 46 up-regulated DEGs in the 20–25 °C condition, and 2253 down-regulated vs 1776 up-regulated DEGs in the 20–30 °C condition (Table 1, Fig. 2e,f). Importantly, 112 DGEs were identified as being shared between both conditions (Fig. 2g).

After annotation and gene name mining filtering, a considerable reduction in the number of DEGs was observed (see Tables 1 and 2). For each condition, 27 and 541 DEGs were collected at 20–25 °C and 20–30 °C, respectively. The annotation sub-selection identified 48 and 1334 DEGs annotated as either hypothetical or uncharacterized proteins in both conditions (see Supplementary Table 1; Tables 1 and 2).

Despite this reduction, the gene mining analyses allowed to identify several stress related-genes in each condition. The 20–25 °C condition revealed four genes annotated as E3 ubiquitin-protein ligase MIB2, one as cytochrome P450 4F6 and one as ATP-dependent RNA helicase A protein in the up-regulated gene set. Additionally, three ubiquitin genes were found in the down-regulated set (see Supplementary Table 1). Differently, the 20–30 °C condition revealed 10 heat shock proteins, two histone H1-delta-like proteins, as well as proteins annotated as "elongation of very long chain fatty acids protein 4-like" among the top 20 up-regulated, as well as five E3 ubiquitin-proteins, two cytochrome P450 2 U1 and three toll-like proteins among the top 20 downregulated genes include (see Supplementary Table 1).

Genes such as the cyclophilin (UpiG00000032055), "cytochrome P450 2C15-like isoform X1" (UpiG00000029957), two heat shock proteins (UpiG00000001087 and UpiG00000032932), two selenoprotein F genes (UpiG00000011648 and UpiG00000011649), one E3 ubiquitin ligase (UpiG00000010051) and one ubiquitin hydrolase (UpiG00000001532) were found down-regulated in both conditions. Conversely, four E3 ubiquitin-protein ligase MIB2 (UpiG00000022863, UpiG00000022861, UpiG00000022862, UpiG00000022859) and one cytochrome P450 (UpiG00000022885) were found to be up-regulated at both challenge temperatures (see Supplementary Table 1).

Enrichment analyses of the southern *U. pictorum* population at 20–25 °Condition revealed multiple BP, CC and MF enriched categories. Only BP and MF categories were identified in the up-regulated gene sets, which were globally linked with lipid metabolism, vascular development, immune signalling, chromosomal organization, metabolic processes, enzymatic activities, redox reactions, and transcriptional regulation (see Supplementary Fig. 3 and Supplementary Table 4). Down-regulated genes were found to be enriched in BP and CC categories. These genes were mainly linked with protein folding, stress responses, reproductive development, endoplasmic reticulum and motility structures (e.g. flagella and cilia) (see Supplementary Fig. 4 and Supplementary Table 5).

Enrichment analyses of 20–30° DEGs also revealed members of the three GO categories (see Supplementary Figs. 5 and 6, and Supplementary Tables 6 and 7). Importantly, the up-regulated genes were mainly associated with GO terms related to protein regulation and signal transduction, while the down-regulated genes were predominantly linked with cilia/flagella assembly and function, intracellular transport, and cytoskeletal organization (see Supplementary Figs. 5 and 6, and Supplementary Tables 6 and 7).

### 3.1.3. *Unio delphinus northern population - north of Portugal*

The northern population of *U. delphinus* showed almost no response to exposure to increasing temperatures. Only one gene was differently expressed at 20–25 °C condition (down-regulated) and functionally characterised as "cathepsin B isoform X1". At 20–30 °C, two genes were differentially expressed (up-regulated), one functionally characterised as a "peptidyl-prolyl cis-trans isomerase B-like" and the other as "uncharacterized protein" (Table 1 and Supplementary Table 1). Given the small number of differently expressed genes, no enrichment analyses were performed for these comparisons.

### 3.1.4. *Unio delphinus southern population - south of Portugal*

In contrast to the experiment on northern individuals, a greater number of differentially expressed genes were identified in the southern population of *U. delphinus*, mainly under 20–30 °C condition (Table 1). While only seven genes were expressed at 20–25 °C, this number substantially increased to 1803 genes at 20–30 °C (Tables 1 and 2). The distribution of up- and down-regulated genes was consistent across both temperatures. At 20–25 °C, three genes were down-regulated, and four were up-regulated, whereas at 20–30 °C, 788 genes were down-regulated and 1015 were up-regulated (see Table 1, Fig. 2a and b). Additionally, four differentially expressed genes were shared between the two tested conditions (see Fig. 3c).

Filtering by annotation reduced the number of differentially expressed genes at 20–25 °C condition from seven to one (see Supplementary Table 1). Conversely, at 20–30 °C, the number of genes decreased by 86.85%, from 1803 to 237 (see Tables 1 and 2). Of this 86% reduction, 655 genes were annotated as either hypothetical or uncharacterized proteins (see Supplementary Table 1).

Regarding stress-related genes, the only one identified at 20–25 °C was found up-regulated and annotated as cytochrome P450 2B1-like (see Supplementary Table 1). A larger number of genes were retained by gene filtering for individuals reared at 20–30 °C, i.e., 237 (see Table 2). The top 20 up-regulated genes at 20–30 °C include five cytochrome P450, three heat shock proteins and two ATP-dependent DNA helicase, among others (see Supplementary Table 1). The top 20 down-regulated genes at 20–30 °C include five heat shock proteins, three allene oxide synthase-lipoxygenase proteins and two Toll-like receptor 4, among others (see Supplementary Table 1).

Four genes were identified and were differentially expressed under both conditions at temperatures of 20–25 °C and 20–30 °C. Two of these genes were up-regulated in both conditions: cytochrome P450 2B1 (UdeG00000005508) and transmembrane protein 45B (UdeG00000010090). Two other genes were down-regulated in both conditions: peptidyl-prolyl cis-trans isomerase B (UdeG00000038795) and centrosomal protein (UdeG00000034408) (see Supplementary Table 1).

In terms of gene ontology enrichment, the genes up-regulated at 20–25 °C were primarily linked to BP and MF categories, including fatty acid metabolism, xenobiotic detoxification, and oxidative enzyme functions, particularly via cytochrome P450-related pathways (see Supplementary Fig. 7 and Supplementary Tables 8). The down-regulated genes at 20–25 °C were associated with three categories and were generally linked with nucleotide metabolism, centrosome function, and redox activity (see Supplementary Fig. 8 and Supplementary Tables 9).

Enrichment analyses of individuals at 20–30 °C were more comprehensive, enabling the identification of down- and up-regulated gene sets in MF, BP and CC (see Supplementary Figs. 9 and 10 and Supplementary Tables 10 and 11). Up-regulated genes were strongly associated with mitochondrial activity, oxidative metabolism, and translation processes, while down-regulated genes were predominantly linked with neuro-immune signalling, ion transport, and membrane receptor activity. (see Supplementary Figs. 9 and 10 and Supplementary Tables 10 and 11).

## 4. Discussion

Freshwater mussels represent the most diverse group of freshwater bivalves, with some species, such as *Unio pictorum* and *Unio delphinus*, thriving in highly contrasting environments (Lopes-Lima et al., 2017). *Unio pictorum* is one of the most widely distributed freshwater mussel species in Europe, ranging from Great Britain to the Ural River in Russia and into Asia. It inhabits a variety of freshwater habitats, including rivers, streams, floodplains and lakes, from diverse environmental regimes, such as the Taiga/Tundra of northern Europe and the Temperate Forest of central and southern parts of the continent (Lopes-Lima et al., 2017; Vinarski et al., 2020). *Unio delphinus* has a much more restricted distribution, being endemic to the western Iberian Peninsula, where it occupies diverse freshwater habitats across all regional climatic regimes, including the northern Temperate Forests and southern Mediterranean dry regimes (Lopes-Lima et al., 2017). Given their sessile lifestyle and the geographically isolated nature, it is plausible that different populations have evolved unique local adaptive strategies, including genetic differentiation and phenotypic plasticity. These adaptations may, in turn, confer population-specific advantages in coping with rapid environmental changes.

In this study, we employed transcriptome-wide gene expression profiling, reflecting the primary mechanism of phenotypic plasticity, to assess how two populations of *Unio* species inhabiting highly contrasting environments respond to a simulated prolonged heat stress. Our findings

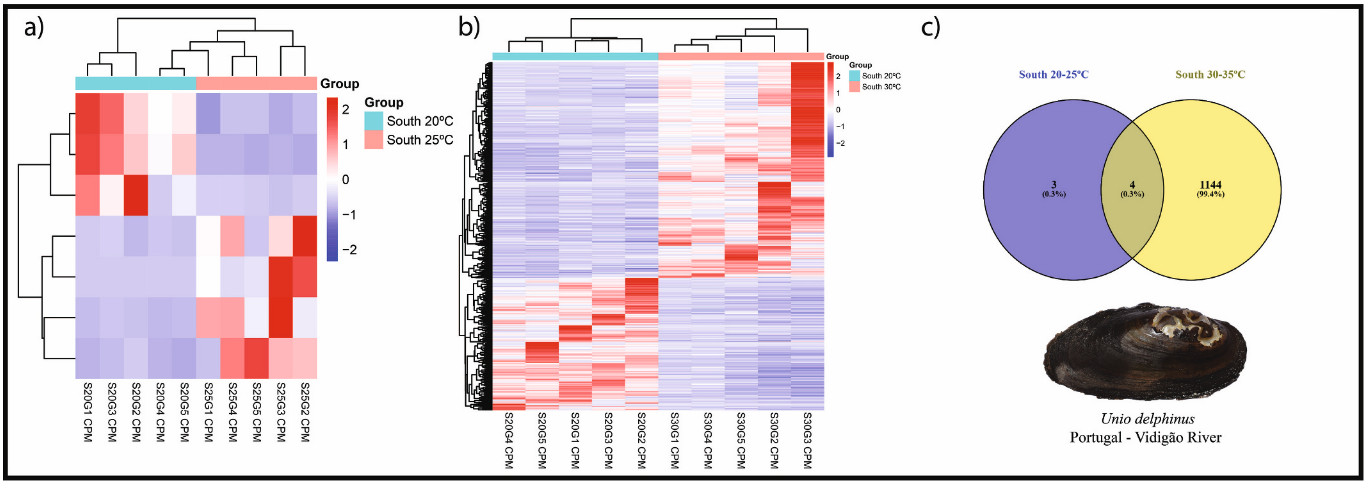


**Fig. 3.** Results of differentially expressed genes analyses among individuals of the southern population (South of Portugal, $n = 15$) of *Unio delphinus* cultured under different temperature conditions. a) Hierarchical Heatmap corresponding to individuals reared at 25 °C compared to the control temperature of 20 °C (South 20 °C ∩ South 25 °C). b) Hierarchical Heatmap corresponding to individuals reared at 30 °C compared to the control temperature of 20 °C (South 20 °C ∩ South 30 °C). c) Venn diagram of reporting the intersection of differentially expressed genes between the two tested conditions. All heatmaps were constructed using the SRPLOT online tool, using the samples' CPM estimations resulting from Degust.

reveal that populations from contrasting environments exhibit markedly different gene expression responses when exposed to identical thermal stress conditions. Furthermore, the functional pathways activated in response to heat stress differ more between populations than between species, suggesting that local environmental history plays a more prominent role than phylogenetic relatedness in shaping thermal response.

### 4.1. *Northern populations have a moderated response to increasing temperatures, with a highlighted role of cytochrome P450s in Unio pictorum*

An emerging pattern from our analysis is that both species' northern populations exhibit a moderated transcriptomic response to increasing temperatures, characterised by a lower number of DEGs. This trend is particularly striking in the northern *U. delphinus* population (North of Portugal), where only three genes were differentially expressed across both temperature comparisons. Despite the low number, two of these genes are functionally associated with stress response pathways. Notably, Cathepsin B, upregulated at 25 °C, is implicated in cellular stress, apoptosis, and protein degradation and has been consistently reported as a heat-responsive gene in multiple organisms (Meistertzheim et al., 2007; Lockwood et al., 2010; Luo et al., 2014; Miao et al., 2015). Similarly, peptidyl-prolyl cis-trans isomerase B, a member of the PPIase gene family downregulated at 30 °C, plays a crucial role in accelerating protein folding (Compton et al., 1992; Schiene-Fischer, 2015).

The northern *U. pictorum* population (Sweden) exhibited a coordinated stress response to increased temperature. Although this response was globally dominated by the expression of cytochrome P450 (CYPs) gene – nine DEGs at 20–25 °C and six at 20–30 °C - several other pathways and additional canonical heat stress response genes were activated as the temperature increased. In addition to these catalysts, several other members of the drug metabolism process were activated and up-regulated, including conjugation enzymes such as glutathione S-transferase and transporters such as ATP-binding cassette (ABC) proteins. At 20–30 °C, this pattern became even clearer, as *U. pictorum* expressed molecular chaperones involved in rescuing misfolded proteins, such as the Hsp90 co-chaperone Cdc37 and the activator of 90 kDa heat shock protein ATPase homolog 1-like. Moreover, it also expressed components involved in degrading damaged or unfolded proteins, such as ubiquitin-conjugating enzyme E2–34 kDa (Supplementary Table 1). Furthermore, several genes that were found downregulated are associated with lysosomal and immune functions, including phospholipid-transporting ATPase ABCA3, cathepsin L1, and peptidoglycan-recognition protein SC2. This trend is further supported by enrichment analyses showing reduced activity in developmental processes, extracellular matrix regulation, and metabolic pathways.

Remarkably, cytochrome P450s (CYPs) are a common denominator in the gene expression of the northern population of *U. pictorum,* indicating a targeted and possibly conserved response. CYPs constitute the largest gene family of enzymes involved in NADPH/$O_2$-dependent hydroxylation reactions across all domains of life and play vital roles in detoxifying both exogenous and endogenous compounds (Dermauw et al., 2020; Pandian et al., 2020). Their involvement in antioxidant activity, fatty acid metabolism, and sterol synthesis makes them central to managing the oxidative and metabolic stress associated with elevated temperatures. Accordingly, their differential expression in response to thermal stress has been reported across various taxa (Huang et al., 2017; Wang et al., 2021a, 2021b; Ashraf et al., 2022; Li et al., 2023). Interestingly, while CYPs have been studied in other bivalves under heat stress (e.g., (Jarque et al., 2014; Robertson et al., 2017; Lima et al., 2018; Dong et al., 2024), their overwhelming representation in *U. pictorum* has not been previously documented and warrants further investigation. At the genomic level, in Unionida, CYPs are also characterised by lineage-specific expansions (Rogers et al., 2025, 2023, 2021). These recent genome studies show that the freshwater mussel species *Megalonaias nervosa*, *Elliptio spinosa*, and *Elliptio crassidens* revealed exceptionally high numbers of CYPs (172, 183, and 183, respectively), far exceeding the number observed in humans ($n = 57$) (Sim and Ingelman-Sundberg, 2010). Consistently, mining the genome annotations of *U. pictorum* and *U. delphinus* revealed a similar number of CYPs (144 and 134, respectively).

Taken together, these results indicate that the northern populations of both species exhibit a conserved pattern of gene expression change in response to elevated temperatures, albeit more subdued than in their southern counterparts. Specifically, in the northern *U. pictorum* population, the early response to moderate heat stress (25 °C) appears to rely heavily on CYPs activity. Only upon exposure to more extreme temperatures (30 °C) does the response broaden to include classical stress-related mechanisms such as chaperones and protein degradation pathways.

Interestingly, CYPs expression is also prominent in the southern populations of both species, reinforcing its potential role as a core component of the thermal stress response. When considered alongside the high copy numbers of CYPs found in the genomes of *U. pictorum* and *U. delphinus*, and similar expansions reported in other distantly related freshwater mussel species, our findings suggest that CYPs may represent rapidly evolving gene families with key roles in local adaptation.

### 4.2. *Southern populations have an intense response to increasing temperatures, relying on protein folding (HSP), ubiquitination, oxidative stress, antioxidation, apoptosis and immune response*

The southern populations of both species exhibited a markedly stronger transcriptomic response to heat stress, particularly in terms of the number of differentially expressed genes (Table 1). This was due to a higher number of DEGs identified in both species. These findings allowed us to highlight and explore several mechanisms not visible in northern populations, mainly in *U. pictorum* at 20–25 °C, and *U. pictorum* and *U. delphinus at* 20–30 °C.

Similar to the pattern observed in the northern populations, *U. delphinus*' southern population (South of Portugal) showed a weaker response than *U. pictorum*'s southern population (Poland), especially at 20–25 °C. Nevertheless, an upregulated cytochrome P450 2B1 could still be identified, underscoring the central importance of the cytochrome P450 family in the thermal stress response. GO terms analysis reinforced this finding by revealing the enrichment of gene ontology (GO) terms related to fatty acid metabolism, xenobiotic detoxification, and oxidoreductase activity, particularly within cytochrome P450-associated pathways (e.g., Epoxygenase P450 pathway and xenobiotic metabolic processes).

This scenario changed in the southern population of *U. pictorum* (Poland) at 20–25 °C. Under this condition, it was possible to identify several gene members linked to drug metabolism. Unlike the northern populations (Sweden), where CYPs appear to be the main driver of thermal response, only two CYPs were found to be differentially expressed in this population at 20–25 °C. The dominant response involved the upregulation of multiple genes annotated as E3 ubiquitin-protein ligase MIB2, which is a key mediator in the ubiquitination process. These findings are consistent with previous studies on freshwater mussels and other bivalves, which have also linked the ubiquitination pathway to the heat stress response (Luo et al., 2014; Li et al., 2017). Ubiquitination is a crucial cellular mechanism that targets irreversibly misfolded or damaged proteins for degradation, thereby preventing the accumulation of cytotoxic aggregates (Ciechanover, 1994; Kültz, 2005; Li et al., 2017). Proteins that cannot be refolded by molecular chaperones are tagged with ubiquitin molecules and subsequently degraded via the 26S proteasome or lysosomal pathways. Further support comes from the enrichment analyses, which indicate a broad adaptive shift in response to stress or external stimuli, involving increased transcriptional and metabolic activity. Key functional pathways include lipid metabolism, immune signalling, oxidoreductase function, and transcriptional regulation. In addition to this process, several molecular chaperones were found to be downregulated at 20–25 °C, including heat shock protein 70, selenoprotein F, T-complex protein 1, cyclophilin, and DnaJ homolog subfamily B member 9 (see Supplementary Table 1). These proteins usually help with protein folding and prevent aggregation under thermal stress (Luo et al., 2014; Li et al., 2017). Their downregulation, coupled with the upregulation of ubiquitin-related genes, suggests a potential change in the cell's strategy, favouring the degradation of misfolded proteins over their stabilisation or refolding.

Enrichment results are consistent with this interpretation, showing a reduced role for protein stress response pathways, particularly those linked to protein folding and endoplasmic reticulum function. Moreover, downregulation of reproductive and motility-related processes, including genes associated with flagella and cilia, is observed. This is particularly relevant given that gills play a central role in freshwater mussel reproduction, serving not only for respiration but also as the site of fertilisation and initial larval development (Bauer, 2001). Although specimens in this study were not gravid, previous research has shown that *U. pictorum* females prematurely release larvae under hypoxic or thermal stress. This behaviour may enhance lifetime reproductive success in long-lived, annually reproducing species (Aldridge and McIvor, 2003). The downregulation of reproductive functions under thermal stress may reflect an adaptive energy-saving strategy, allowing individuals to prioritise immediate survival over reproduction in the face of environmental challenges.

The southern population of both species exposed to 30 °C exhibited the highest number of DEGs (Table 1).

*Unio delphinus* showed a substantially lower response compared to *U. pictorum*, with 1803 DEGs, approximately 45% fewer than the 4029 DEGs identified in *U. pictorum*. Notably, this DEG count for *U. pictorum* exceeds those reported in previous studies under similar thermal conditions (e.g., 1934 in *Villosa lienosa* at 29 ± 2 °C, 2559 in *Pyganodon grandis*, and 2532 in *Uniomerus tetralasmus* at 33 °C) (Luo et al., 2014; Wang et al., 2015), despite the inclusion of desiccation stress in the latter two.

Importantly, under the 20–30 °C conditions, CYPs remerge as a relevant pattern in response to increased temperature. Overall, filtering revealed 541 DEGs in *U. pictorum* and 237 in *U. delphinus*. Of these, 17 in *U. pictorum* and 14 in *U. delphinus* were annotated as cytochrome P450s. Five CYPs were identified among the top 20 most highly expressed genes in *U. delphinus*, including *CYP2H1*, *CYP2B1*, and *CYP2B4*. In contrast, none of the top 20 DEGs in *U. pictorum* genes displayed CYPs, although other CYPSs (e.g., *CYP2U1* and *CYP1A*), were identified among the remaining genes.

Both species showed strong expression of heat shock proteins (HSPs), particularly members of the HSP70 family. Genome annotations revealed 330 and 328 HSP70 gene copies in *U. pictorum* and *U. delphinus*, respectively, substantially higher than in other freshwater mussels (e.g., *M. nervosa* with 96; *E. spinosa* with 153; *E. crassidens* with 210) (Rogers et al., 2025, 2023, 2021). At 30 °C, 42 HSP70s were differentially expressed in *U. pictorum* and 12 in *U. delphinus*, with most being downregulated. Notably, *HSP70 B2* and *HSP27* were among the most highly upregulated genes in both species. *U. pictorum* also showed strong upregulation of *HSP68*. Interestingly, several *HSP70-12 A* and *HSP70-12B* paralogs, unique in their nucleotide-binding domains, were downregulated, consistent with prior findings in *P. grandis* and *U. tetralasmus* (Luo et al., 2014). Considering these genes demonstrated expression in atherosclerotic lesions in mammals (Han et al., 2003; Radons, 2016), they might play a role in the redeployment of fatty acid metabolism or muscle membrane structure during heat stress. Additionally, multiple HSP40 (DnaJ) genes were differentially expressed, nine in *U. pictorum* and three in *U. delphinus*. While *U. delphinus* showed consistent upregulation (e.g., *dnaJ B11*, *C1*, *C30*), *U. pictorum* had both up- and downregulated DnaJ genes. The observed expression patterns closely align with previous findings in other freshwater and marine bivalves (Luo et al., 2014; Li et al., 2017). Additional chaperones such as alpha-crystallin B, calnexin, SIP1, and ASHA1 were also upregulated, consistent with known protective roles in protein stability under stress.

Genes involved in ubiquitination, particularly E3 ubiquitin ligases, showed species-specific expression patterns. *U. pictorum* had 31 up- and 28 downregulated E3 ligases, while *U. delphinus* had 5 up- and 10 downregulated. Notably, *MIB2* was upregulated in *U. pictorum* (five copies) but downregulated in *U. delphinus* (one copy), echoing contrasting responses reported in *P. grandis* and *U. tetralasmus* (Luo et al., 2014). GO enrichment confirmed ubiquitination as a major functional category in *U. pictorum* only.

Differential expression of mitochondrial and metabolic genes suggests species-specific energy responses. Both species expressed multiple *cytochrome c*, *NADH dehydrogenase*, and *secretory phospholipase A2* genes, but *U. delphinus* additionally expressed known heat-responsive genes, including *cytochrome b*, *glutamine synthetase*, and *alternative oxidase*. Enrichment analyses pointed to heightened oxidative metabolism and respiratory activity in *U. delphinus*, potentially reflecting adaptation to southern Iberian dry-season conditions characterised by extreme heat and low water availability (Belmar et al., 2019; Bonada et al., 2020). The southern rivers of the Iberian Peninsula are characterised by water flow cessation during the dry season, concentrating freshwater taxa in small ponds (Belmar et al., 2019; Bonada et al., 2020). Conversely, physicochemical conditions, such as $O_2$ and nutrient concentration, are largely altered. It is likely that through local adaptive mechanisms, the southern population of *U. delphinus* might respond to increasing temperatures by triggering an acute bioenergetic and metabolic molecular machinery in anticipation of the harsh conditions of the dry seasons.

Several indicators of DNA damage and cell apoptosis were also shared between the two species, *GADD45α*, *caspases 7, 8, 9*, *calpain-A*, and *TRAF2/4*. Both species upregulated multiple inhibitors of apoptosis proteins (IAPs) and E2 ubiquitin-conjugating enzymes, several among the most highly expressed genes. These mechanisms are consistent with heat-tolerant species such as *U. tetralasmus*, where IAPs mediate cell survival via ubiquitin signalling (Syed et al., 2006; Stempin et al., 2011; Silke and Meier, 2013).

Immune-related genes such as *NF-kappa-B inhibitors*, *TLRs*, *big defensin*, and *TBK1* were differentially expressed in both species as well. *U. pictorum* uniquely expressed *C-type lectin*, *IRE1*, and *histone H4*, while *U. delphinus* showed expression of *Complement C3*, *interferon regulatory factors*, and *lysozyme 1*. These genes are central to humoral immunity and inflammatory regulation, particularly under environmental stress (Ghosh et al., 1998; Lin et al., 2000).

Thermal stress induced expression changes in cytoskeleton-related genes. Shared genes included *microtubule-associated proteins*, *tubulins*, and *protocadherins*, with *U. pictorum* expressing a broader set, including *collagen chains*, *fibrillin*, and *myosin heavy chain kinase B*. These genes, mostly downregulated, support known cytoskeletal reorganisation patterns under thermal stress (Evans and Hofmann, 2012; Luo et al., 2014). GO enrichment confirmed stronger cytoskeletal reconfiguration in *U. pictorum*.

In summary, the southern population of both species exhibit complex, multi-layered molecular responses to extreme temperatures, with *U. pictorum* (Poland) showing a more intense, proteostasis- and detoxification-centred profile, and *U. delphinus* (South of Portugal) displaying a bioenergetically driven response suggestive of local adaptation to Mediterranean-like climates. The roles of CYPs, HSPs, and ubiquitination pathways emerge as central, but the divergence in gene expression patterns, even between congeneric species, underscores the importance of considering population-level plasticity in conservation and resilience assessments.

## 5. Conclusion

The findings of this study clearly suggest population-specific local adaptations, potentially enabling these species to persist across a broad range of environmental conditions. Future research should expand to include more populations across each species' distribution, enabling the detection of adaptive clines and population-level thermal thresholds. In addition, investigating adaptive polymorphisms and epigenetic modifications will be key to identifying the regulatory bases of plasticity and stress response. Such knowledge is vital for anticipating how different populations may respond to climate change and for designing targeted, effective conservation strategies. In the face of accelerating global warming, preserving not just species but the evolutionary potential within them is the best defence against biodiversity loss.

## Ethics and permit approval statement

This work has been approved by the CIIMAR ethical committee and by CIIMAR Managing Animal Welfare Body (ORBEA) according to the European Union Directive 2010/63/EU. During the preparation of this manuscript, the authors utilized ChatGPT-4 to enhance readability and correct grammar. All content was thoroughly reviewed and edited by the authors, who take full responsibility for the accuracy and integrity of the published article.

## CRediT authorship contribution statement

**André Gomes-dos-Santos:** Writing – review & editing, Writing – original draft, Software, Resources, Methodology, Formal analysis, Data curation, Conceptualization. **Manuel Lopes-Lima:** Writing – review & editing, Writing – original draft, Validation, Supervision, Formal analysis, Conceptualization. **Beatriz Silva:** Writing – review & editing, Methodology, Formal analysis, Data curation. **André M. Machado:** Writing – review & editing, Software, Methodology, Data curation. **Rui Pinto:** Writing – review & editing, Software, Methodology, Data curation. **Martin Österling:** Writing – review & editing, Validation, Resources, Investigation, Formal analysis. **Magnus Lovén Wallerius:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **Maria Urbańska:** Writing – review & editing, Validation, Investigation, Data curation. **Janusz Golski:** Writing – review & editing, Resources, Investigation, Formal analysis. **Sławomir Runowski:** Writing – review & editing, Resources, Investigation, Formal analysis. **Sandra Kaźmierczak:** Writing – review & editing, Resources, Investigation, Data curation. **Amílcar Teixeira:** Writing – review & editing, Resources, Investigation, Formal analysis. **Ronaldo Sousa:** Writing – review & editing, Validation, Resources, Investigation, Formal analysis, Data curation. **L. Filipe C. Castro:** Writing – review & editing, Writing – original draft, Validation, Supervision, Formal analysis, Conceptualization. **Paulo Castro:** Writing – review & editing, Resources, Investigation, Data curation. **Francisco Carvalho:** Writing – review & editing, Resources, Investigation, Data curation. **Elza Fonseca:** Writing – review & editing, Software, Methodology, Investigation, Data curation. **Elsa Froufe:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

## Funding

This research was developed under the project EdgeOmics - Freshwater Bivalves at the Edge: Adaptation genomics under climate-change scenarios (PTDC/CTA-AMB/3065/2020) funded by Fundação para a Ciência e a Tecnologia (FCT) through national funds. Fundação para a Ciência e a Tecnologia (FCT) also supported AGS (2023.07625.CEECIND/CP2848/CT0010 and DOI identifier https://doi.org/10.54499/2023.07625.CEECIND/CP2848/CT0010), EF (CEECINST/00027/2021/CP2789/CT0003 and DOI identifier https://doi.org/10.54499/CEECINST/00027/2021/CP2789/CT0003. FCT also provided funds within the scope of the Strategic Funding UIDB/04423/2020 (https://doi.org/10.54499/UIDB/04423/2020), UIDP/04423/2020 (https://doi.org/10.54499/UIDP/04423/2020), and LA/P/0101/2020 (https://doi.org/10.54499/LA/P/0101/2020).

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

We thank the three anonymous reviewers and the editor for the helpful remarks and suggestions, which have improved the manuscript.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbd.2026.101778.

## Data availability

The raw reads sequencing outputs were deposited at the NCBI Sequence Read Archive under BioProject accession PRJNA128314, accessible through https://www.ncbi.nlm.nih.gov/sra/PRJNA1283142. All metadata generated from the Differential Gene Expression analyses is provided as Supplementary Material (Files, Figures and Tables) of the manuscript. All software with respective versions and parameters used for producing the resources presented here are listed in the methods section. Software programs with no parameters associated were used with the default settings.

## References

Aldridge, D.C., McIvor, A.L., 2003. Gill evacuation and release of glochidia by unio pictorum and unio tumidus (bivalvia: unionidae) under thermal and hypoxic stress. J. Molluscan Stud. 69, 55–59.

Aldridge, D.C., et al., 2022. Freshwater mussel conservation: a global horizon scan of emerging threats and opportunities. Glob. Chang. Biol. 00, 1–15.

Ashraf, H.J., et al., 2022. Comparative transcriptome analysis of Tamarixia radiata (Hymenoptera: Eulophidae) reveals differentially expressed genes upon heat shock. Comp. Biochem. Physiol. Part D Genomics Proteomics 41, 100940.

Babicki, S., et al., 2016. Heatmapper: web-enabled heat mapping for all. Nucleic Acids Res. 44, W147–W153.

Bauer, G., 2001. Ecology and Evolution of the Freshwater Mussels Unionoida, vol. 145. Springer Science & Business Media.

Belmar, O., et al., 2019. Functional responses of aquatic macroinvertebrates to flow regulation are shaped by natural flow intermittence in Mediterranean streams. Freshw. Biol. 64, 1064–1077.

Beninger, P.G., St-Jean, S., Poussart, Y., Ward, J.E., 1993. Gill function and mucocyte distribution in Placopecten magellanicus and Mytilus edulis (Mollusca: Bivalvia): the role of mucus in particle transport. Mar. Ecol. Prog. Ser. 98, 275–282.

Bonada, N., et al., 2020. Conservation and management of isolated pools in temporary rivers. Water (Basel) 12, 2870.

Camacho, C., et al., 2009. BLAST+: architecture and applications. BMC Bioinformatics 10, 1–9.

Capon, S.J., Stewart-Koster, B., Bunn, S.E., 2021. Future of freshwater ecosystems in a 1.5°C warmer world. Front. Environ. Sci. 9.

Ciechanover, A., 1994. The ubiquitin-proteasome proteolytic pathway. Cell 79, 13–21.

Compton, L.A., Davis, J.M., Macdonad, J.R., Bächinger, H.P., 1992. Structural and functional characterization of Escherichia coli peptidyl-prolyl cis-trans isomerases. Eur. J. Biochem. 206, 927–934.

Cushway, K.C., Geist, J., Schwalb, A.N., 2025. Surviving global change: a review of the impacts of drought and dewatering on freshwater mussels. Biol. Rev. 100, 275–307.

da Silva, J.P., et al., 2022. Predicting climatic threats to an endangered freshwater mussel in Europe: the need to account for fish hosts. Freshw. Biol. 67, 842–856.

da Silva, J.P., et al., 2023. Streams in the Mediterranean region are not for mussels: predicting extinctions and range contractions under future climate change. Sci. Total Environ. 883, 163689.

da Silva, J.P., et al., 2024. The role of connectivity in conservation planning for species with obligatory interactions: prospects for future climate scenarios. Glob. Chang. Biol. 30.

Dermauw, W., Van Leeuwen, T., Feyereisen, R., 2020. Diversity and evolution of the P450 family in arthropods. Insect Biochem. Mol. Biol. 127, 103490.

Ding, Y., Zhou, Q., Wang, W., 2012. Origins of new genes and evolution of their novel functions. Annu. Rev. Ecol. Evol. Syst. 43, 345–363. https://doi.org/10.1146/annurev-ecolsys-110411-160513.

Dong, L., Yu, J., Wang, H., Yue, X., Liu, B., 2024. Transcriptomic insights into vibrio-induced mortality in the clam Meretrix petechialis under high temperature. Comp. Biochem. Physiol. Part D Genomics Proteomics 50, 101226.

Duncan, E.J., Gluckman, P.D., Dearden, P.K., 2014. Epigenetics, plasticity, and evolution: how do we link epigenetic change to phenotype? J. Exp. Zool. B Mol. Dev. Evol. 322, 208–220.

Evans, T.G., Hofmann, G.E., 2012. Defining the limits of physiological plasticity: how gene expression can assess and predict the consequences of ocean change. Philos. Trans. R. Soc. B 367, 1733–1745.

Froufe, E., et al., 2014. Genetic diversity of the pan-European freshwater mussel *Anodonta anatina* (Bivalvia: Unionoida) based on CO1: new phylogenetic insights and implications for conservation. Aquat. Conserv. 24, 561–574.

Froufe, E., et al., 2016. Phylogeny, phylogeography, and evolution in the Mediterranean region: news from a freshwater mussel (*Potomida*, Unionida). Mol. Phylogenet. Evol. 100, 322–332.

Ganser, A.M., Newton, T.J., Haro, R.J., 2015. Effects of elevated water temperature on physiological responses in adult freshwater mussels. Freshw. Biol. 60, 1705–1716.

Ge, S.X., Jung, D., Yao, R., 2020. ShinyGO: a graphical gene-set enrichment tool for animals and plants. Bioinformatics 36, 2628–2629.

Gerdol, M., et al., 2020. Massive gene presence-absence variation shapes an open pan-genome in the Mediterranean mussel. Genome Biol. 21, 275.

Ghalambor, C.K., et al., 2015. Non-adaptive plasticity potentiates rapid adaptive evolution of gene expression in nature. Nature 525, 372–375.

Ghosh, S., May, M.J., Kopp, E.B., 1998. NF-κB and rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. 16, 225–260.

Gomes-dos-Santos, A., et al., 2023a. A PacBio hi-fi genome assembly of the painter's mussel *Unio pictorum* (Linnaeus, 1758). Genome Biol. Evol. https://doi.org/10.1093/gbe/evad116.

Gomes-dos-Santos, A., et al., 2023b. Pacbio hi-fi genome assembly of the Iberian dolphin freshwater mussel *Unio delphinus* Spengler, 1793. Sci. Data 10, 340.

Gough, H.M., Gascho Landis, A.M., Stoeckel, J.A., 2012. Behaviour and physiology are linked in the responses of freshwater mussels to drought. Freshw. Biol. 57, 2356–2366.

Graf, D.L., Cummings, K.S., 2006. Palaeoheterodont diversity (Mollusca: Trigonioida + Unionoida): what we know and what we wish we knew about freshwater mussel evolution. Zool. J. Linnean Soc. 148, 343–394.

Graf, D.L., Cummings, K.S., 2007. Review of the systematics and global diversity of freshwater mussel species (Bivalvia: Unionoida). J. Molluscan Stud. 73, 291–314. Preprint at. https://doi.org/10.1093/mollus/eym029.

Graf, D.L., Cummings, K.S., 2022. The freshwater mussels (Unionoida) of the world (and other less consequential bivalves). *http://www.mussel-project.net/*.

Han, Z., Truong, Q.A., Park, S., Breslow, J.L., 2003. Two Hsp70 family members expressed in atherosclerotic lesions. Proc. Natl. Acad. Sci. 100, 1256–1261. https://doi.org/10.1073/PNAS.252764399.

Huang, H., et al., 2017. Comparative analysis of the transcriptional responses to low and high temperatures in three rice planthopper species. Mol. Ecol. 26, 2726–2737.

IPCC, 2023. Climate change 2023: synthesis report. In: Contribution of Working Groups I, II and III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change. IPCC, Geneva, Switzerland. https://doi.org/10.59327/IPCC/AR6-9789291691647 [Core Writing Team, H. Lee and J. Romero (eds.)]. 184 pp.

Jarque, S., et al., 2014. Seasonal variations of gene expression biomarkers in Mytilus galloprovincialis cultured populations: temperature, oxidative stress and reproductive cycle as major modulators. Sci. Total Environ. 499, 363–372.

Jørgensen, C.B., 1974. On gill function in the mussel *Mytilus Edulis* L. Ophelia 13, 187–232.

Kelly, M., 2019. Adaptation to climate change through genetic accommodation and assimilation of plastic phenotypes. Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 374, 20180176.

Keogh, S.M., et al., 2025. Secondary contact erodes Pleistocene diversification in a wide-ranging freshwater mussel *(Quadrula*). Mol. Ecol. 34.

Kültz, D., 2005. Molecular and evolutionary basis of the cellular stress response. Annu. Rev. Physiol. 67, 225–257.

Lang, R.P., Bayne, C.J., Camara, M.D., Cunningham, C., Jenny, M.J., Langdon, C.J., 2009. Transcriptome profiling of selectively bred pacific oyster Crassostrea gigas families that differ in tolerance of heat shock. Mar. Biotechnol. 11, 650–668. https://doi.org/10.1007/S10126-009-9181-6/TABLES/5.

Le Luyer, J., et al., 2022. Gene expression plasticity, genetic variation and fatty acid remodelling in divergent populations of a tropical bivalve species. J. Anim. Ecol. 91, 1196–1208.

Li, J., Zhang, Yuehuan, Mao, F., Tong, Y., Liu, Y., Zhang, Yang, Yu, Z., 2017. Characterization and identification of differentially expressed genes involved in thermal adaptation of the Hong Kong oyster Crassostrea hongkongensis by digital gene expression profiling. Front. Mar. Sci. 4. https://doi.org/10.3389/fmars.2017.00112.

Li, W., et al., 2023. The role of cytochrome P450 3A2 and 4V2 in response to high-temperature stress in Tetranychus truncatus (Acari: Tetranychidae). Exp. Appl. Acarol. 91, 263–277.

Lima, D., et al., 2018. Molecular and cellular effects of temperature in oysters *Crassostrea brasiliana* exposed to phenanthrene. Chemosphere 209, 307–318.

Lin, P.H., Huang, L.H., Steward, R., 2000. Cactin, a conserved protein that interacts with the Drosophila IκB protein Cactus and modulates its function. Mech. Dev. 94, 57–65.

Lockwood, B.L., Sanders, J.G., Somero, G.N., 2010. Transcriptomic responses to heat stress in invasive and native blue mussels (genus *Mytilus*): molecular correlates of invasive success. J. Exp. Biol. 213, 3548–3558. https://doi.org/10.1242/jeb.046094.

Lopes-Lima, M., et al., 2017. Conservation status of freshwater mussels in Europe: state of the art and future challenges. Biol. Rev. 92, 572–607.

Lopes-Lima, M., et al., 2018. Conservation of freshwater bivalves at the global scale: diversity, threats and research needs. Hydrobiologia 810, 1–14.

Lopes-Lima, M., et al., 2021. Major shortfalls impairing knowledge and conservation of freshwater molluscs. Hydrobiologia 848 (12), 2831–2867. **848**. 2021.

Luo, Y., et al., 2014. Transcriptomic profiling of differential responses to drought in two freshwater mussel species, the Giant floater Pyganodon grandis and the Pondhorn Uniomerus tetralasmus. PLoS One 9, e89481.

Markovic, D., Carrizo, S.F., Kärcher, O., Walz, A., David, J.N.W., 2017. Vulnerability of European freshwater catchments to climate change. Glob. Chang. Biol. 23, 3567–3580.

McDowell, W.G., Sousa, R., 2019. Mass mortality events of invasive freshwater bivalves: current understanding and potential directions for future research. Front. Ecol. Evol. 7.

Meistertzheim, A.L., Tanguy, A., Moraga, D., Thébault, M.T., 2007. Identification of differentially expressed genes of the Pacific oyster Crassostrea gigas exposed to prolonged thermal stress. FEBS J. 274, 6392–6402. https://doi.org/10.1111/J.1742-4658.2007.06156.X;JOURNAL:JOURNAL:14321033.

Miao, J., Chi, L., Pan, L., Song, Y., 2015. Generally detected genes in comparative transcriptomics in bivalves: toward the identification of molecular markers of cellular stress response. Environ. Toxicol. Pharmacol. 39, 475–481.

Nogueira, J.G., Lopes-Lima, M., Varandas, S., Teixeira, A., Sousa, R., 2021. Effects of an extreme drought on the endangered pearl mussel Margaritifera margaritifera: a before/after assessment. Hydrobiologia 848, 3003–3013.

Pandian, B.A., Sathishraj, R., Djanaguiraman, M., Prasad, P.V.V., Jugulam, M., 2020. Role of cytochrome P450 enzymes in plant stress response. Antioxidants 9, 454.

Pourmozaffar, S., et al., 2020. The role of salinity in physiological responses of bivalves. Rev. Aquac. 12, 1548–1566.

Radons, J., 2016. The human HSP70 family of chaperones: where do we stand? Cell Stress Chaperones 21, 379–404. https://doi.org/10.1007/S12192-016-0676-6.

Rivera, H.E., et al., 2021. A framework for understanding gene expression plasticity and its influence on stress tolerance. Mol. Ecol. 30, 1381–1397.

Robertson, L.S., Galbraith, H.S., Iwanowicz, D., Blakeslee, C.J., Cornman, R.S., 2017. RNA sequencing analysis of transcriptional change in the freshwater mussel Elliptio complanata after environmentally relevant sodium chloride exposure. Environ. Toxicol. Chem. 36, 2352–2366. https://doi.org/10.1002/ETC.3774.

Robinson, M.D., Oshlack, A., 2010. A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biol. 11 (3). https://doi.org/10.1186/GB-2010-11-3-R25, 2010. 11, R25-.

Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2010. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140. https://doi.org/10.1093/bioinformatics/btp616.

Rogers, R.L., et al., 2021. Gene family amplification facilitates adaptation in freshwater unionid bivalve *Megalonaias nervosa*. Mol. Ecol. 30, 1155–1173.

Rogers, R.L., Grizzard, S.L., Garner, J.T., 2023. Strong, recent selective sweeps reshape genetic diversity in freshwater bivalve *Megalonaias nervosa*. Mol. Biol. Evol. 40.

Rogers, R.L., Wares, J.P., Garner, J.T., 2025. Genome Evolution in an Endangered Freshwater Mussel.

Roznere, I., Sinn, B.T., Daly, M., Watters, G.T., 2021. Freshwater mussels (Unionidae) brought into captivity exhibit up-regulation of genes involved in stress and energy metabolism. Sci. Rep. 11, 2241. https://doi.org/10.1038/s41598-021-81856-7.

Sayer, C.A., et al., 2025. One-quarter of freshwater fauna threatened with extinction. Nature 2025, 1–8. https://doi.org/10.1038/s41586-024-08375-z.

Scheiner, S.M., 1993. Genetics and evolution of phenotypic plasticity. Annu. Rev. Ecol. Syst. 24, 35–68.

Schiene-Fischer, C., 2015. Multidomain peptidyl prolyl cis/trans isomerases. Biochim. Biophys. Acta Gen. Subj. 1850, 2005–2016.

Silke, J., Meier, P., 2013. Inhibitor of apoptosis (IAP) proteins–modulators of cell death and inflammation. Cold Spring Harb. Perspect. Biol. 5, a008730.

Sim, S.C., Ingelman-Sundberg, M., 2010. The human cytochrome P450 (CYP) allele nomenclature website: a peer-reviewed database of CYP variants and their associated effects. Hum. Genomics 4, 278–281.

Sousa, R., et al., 2018. Die-offs of the endangered pearl mussel *Margaritifera margaritifera* during an extreme drought. Aquat. Conserv. 28, 1244–1248.

Sousa, R., et al., 2023. A roadmap for the conservation of freshwater mussels in Europe. Conserv. Biol. 37, e13994.

Stempin, C.C., et al., 2011. The E3 ubiquitin ligase mind bomb-2 (MIB2) protein controls B-cell CLL/lymphoma 10 (BCL10)-dependent NF-κB activation. J. Biol. Chem. 286, 37147–37157.

Syed, N.A., Andersen, P.L., Warrington, R.C., Xiao, W., 2006. Uev1A, a ubiquitin conjugating enzyme variant, inhibits stress-induced apoptosis through NF-κB activation. Apoptosis 11, 2147–2157.

Tankersley, R.A., 1996. Multipurpose gills: effect of larval brooding on the feeding physiology of freshwater Unionid mussels. Invertebr. Biol. 115, 243.

Tomanek, L., Zuzow, M.J., 2010. The proteomic response of the mussel congeners Mytilus galloprovincialis and M. trossulus to acute heat stress: implications for thermal tolerance limits and metabolic costs of thermal stress. J. Exp. Biol. 213, 3559–3574. https://doi.org/10.1242/JEB.041228.

Vinarski, M.V., et al., 2020. Freshwater mollusca of the circumpolar Arctic: a review on their taxonomy, diversity and biogeography. Hydrobiologia 1–28. https://doi.org/10.1007/s10750-020-04270-6.

Wacker, S., et al., 2024. Resilience of juvenile freshwater pearl mussels to thermal stress. Ecol. Evol. 14.

Wang, R., et al., 2015. Rapid development of molecular resources for a freshwater mussel, *Villosa lienosa* (Bivalvia:Unionidae), using an RNA-seq-based approach, 31, 695–708. https://doi.org/10.1899/11-149.1.

Wang, Y.-C., et al., 2021. High temperature stress induces expression of CYP450 genes and contributes to insecticide tolerance in *Liriomyza trifolii*. Pestic. Biochem. Physiol. 174, 104826.

Zhang, Guofan, Fang, X., Guo, X., Li, L., Luo, R., Xu, F., Yang, P., Zhang, L., Wang, X., Qi, H., Xiong, Z., Que, H., Xie, Y., Holland, P.W.H., Paps, J., Zhu, Y., Wu, F., Chen, Y., Wang, Jiafeng, Peng, C., Meng, J., Yang, L., Liu, J., Wen, B., Zhang, N., Huang, Z., Zhu, Q., Feng, Y., Mount, A., Hedgecock, D., Xu, Z., Liu, Y., Domazet-Lošo, T., Du, Y., Sun, X., Zhang, Shoudu, Liu, B., Cheng, P., Jiang, X., Li, J., Fan, D., Wang, W., Fu, W., Wang, T., Wang, B., Zhang, J., Peng, Z., Li, Yingxiang, Li, Na, Wang, Jinpeng, Chen, M., He, Y., Tan, F., Song, X., Zheng, Q., Huang, R., Yang, Hailong, Du, X., Chen, L., Yang, M., Gaffney, P.M., Wang, S., Luo, L., She, Z., Ming, Y., Huang, W., Zhang, Shu, Huang, B., Zhang, Y., Qu, T., Ni, P., Miao, G., Wang, Junyi, Wang, Q., Steinberg, C.E.W., Wang, H., Li, Ning, Qian, L., Zhang, Guojie, Li, Yingrui, Yang, Huanming, Liu, X., Wang, Jian, Yin, Y., Wang, Jun, 2012. The oyster genome reveals stress adaptation and complexity of shell formation. Nature 490, 49–54. https://doi.org/10.1038/nature11413.