

Hiding from heat: The transcriptomic response of two clam species is modulated by behaviour and habitat

Daniel García-Souto^{a,b}, Víctor Martínez-Mariño^c, Paloma Morán^c, Celia Olabarria^d, Elsa Vázquez^{d,*}

^a Genomas y Enfermedad, Centro Singular de Investigación en Medicina Molecular y Enfermedades Crónicas, Universidad de Santiago de Compostela, 15782, Santiago de Compostela, Spain

^b Departamento de Zoología, Genética y Antropología Física, Universidad de Santiago de Compostela, 15782, Santiago de Compostela, Spain

^c Centro de Investigación Mariña (CIM) and Departamento de Bioquímica, Xenética e Inmunoloxía, Facultade de Bioloxía, Universidade de Vigo, 36310, Vigo, Spain

^d Centro de Investigación Mariña (CIM) and Departamento de Ecoloxía e Bioloxía Animal, Facultade de Ciencias do Mar, Universidade de Vigo, 36310, Vigo, Spain

ARTICLE INFO

Keywords:

Heat stress

Ruditapes sp.

Seagrass

Behavioural response

Transcriptome

Shellfish management

ABSTRACT

Rising occurrence of extreme warming events are profoundly impacting ecosystems, altering their functioning and services with significant socio-economic consequences. Particularly susceptible to heatwaves are intertidal shellfish beds, located in estuarine areas already stressed by factors such as rainfall events, red tides, eutrophication, and pollution. In Galicia, Northwestern Spain, these beds support vital shellfisheries, featuring the native clam *Ruditapes decussatus* and the non-indigenous *R. philippinarum*. Over recent decades, these populations have experienced notable abundance shifts due to various anthropogenic impacts, including climate change. In this habitat, patches of the seagrass *Zostera noltei* that coexist with bare sand can act as thermal refuges for benthic organisms such as clams. To assess the impact of heatwaves on these ecosystems, a mesocosm experiment was conducted. Juveniles of both clam species in two habitat types—bare sand and sand with *Z. noltei*—were exposed to simulated atmospheric heatwaves during diurnal low tide for four consecutive days. Subsequent transcriptomic analysis revealed that high temperatures had a more pronounced impact on the transcriptome of *R. philippinarum* compared to *R. decussatus*. The habitat type played a crucial role in mitigating heat stress in *R. philippinarum*, with the presence of *Z. noltei* notably ameliorating the transcriptomic response. These findings have direct applications in shellfishery management, emphasizing the importance of preserving undisturbed patches of *Z. noltei* as thermal refuges, contributing to the mitigation of heatwave effects on shellfish populations.

1. Introduction

Discrete extreme warming events, i.e. heatwaves, constitute a natural component of climate variability worldwide (e.g. Hart and Scheibling 1988), but they are becoming increasingly common due to global warming (Perkins et al., 2012; Coumou and Rahmstorf 2012; Oliver et al., 2018). Climate projections indicate that heatwaves will become more frequent, more intense and longer lasting throughout the 21st Century (IPCC et al., 2021). Some such events have already occurred, as record atmospheric heatwaves were recorded in Europe in 2003 and 2017 (Lhotka and Kyselý 2022), with notable impacts on ecological systems, human health and economy. A more complete understanding of the biological and ecological impacts of these events is needed to better

predict and adapt to increased heatwave activity in the near future, as such events can drive unprecedented ecosystem change (Smith et al., 2022).

Temperature has a key influence on all physiological processes and thus drives the ecological performance of organisms (e.g. growth, reproduction, phenology and survival) (Seibel and Drazen 2007). Organisms maximise their performance at an optimal environmental range and can survive for short periods in environmental conditions that exceed a threshold in their critical tolerance limits. However, exceeding these temperature limits can lead to negative responses in organisms, varying from sublethal effects on fundamental physiological processes to mortality (He et al., 2022; Masanja et al., 2022; Smith et al., 2022). Sessile or sedentary organisms that cannot escape from the extreme

* Corresponding author.

E-mail addresses: daniel.garcia.souto@usc.es (D. García-Souto), victor.martinez.marinho@gmail.com (V. Martínez-Mariño), paloma@uvigo.es (P. Morán), colabarria@uvigo.gal (C. Olabarria), eotero@uvigo.es (E. Vázquez).

<https://doi.org/10.1016/j.jtherbio.2023.103776>

Received 18 September 2023; Received in revised form 8 December 2023; Accepted 9 December 2023

Available online 20 December 2023

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environmental conditions are particularly prone to extreme thermal stress, e.g. mass mortalities in the Mediterranean Sea (Garrabou et al., 2022), the North East Pacific coast (Harley, 2008) and Australia (Wernberg et al., 2013). Some of these organisms can modify their behavioural responses (Macho et al., 2016; Domínguez et al., 2021; Zhou et al., 2022) or utilise metabolic depression, anaerobic energy production and diverse stress protection mechanisms, including overexpression or underexpression of certain proteins, to provide protection against extreme temperatures (e.g. Pörtner and Farrell 2008; Marshall and McQuaid 2011; Olabarria et al., 2016; He et al., 2022; Masanja et al., 2022; Peruzza et al., 2023). In addition, sessile organisms may minimise thermal stress by interacting with other organisms, i.e. facilitators, that ameliorate stress (Lathlean et al., 2016; Olabarria et al., 2016; Román et al., 2020).

Intertidal areas are particularly vulnerable to the effects of heatwaves as the thermal environment varies widely over the tidal cycle and organisms are frequently exposed to conditions close to their physiological limits (Helmuth et al., 2006). The impacts of heatwaves on organisms inhabiting estuarine areas are of special concern, especially when the affected species support important fisheries or aquaculture such as bivalve fisheries (Domínguez et al., 2021; Masanja et al., 2022; Peruzza et al., 2023).

In Galicia (NW Spain), intertidal areas support important shellfisheries of the native clam *Ruditapes decussatus* (Linnaeus 1758) and the introduced species *R. philippinarum* (Adams and Reeve, 1850), which together provided an income of approx. 33 million € in 2022, involving around 7100 fishers (<http://www.pescadegalicia.gal>). Small-scale Galician shellfisheries, as well as the associated seagrass beds, i.e. *Zostera noltei* Hornemann and *Z. marina* Linnaeus, have experienced important changes in the last few decades due to the presence of diverse anthropogenic stressors, including climate change (Román et al., 2020; Villasante et al., 2022). These shellfish beds are very vulnerable to ocean warming and heatwaves, especially during summer, as they are located in the shallow inner areas of rias, estuarine environments of high tidal amplitude (4 m) exposed for long periods to air temperatures and solar radiation during low tides (Alvarez et al., 2005). A recent study has suggested that ocean warming may threaten these fisheries by modifying the geographic distribution of the species, with a decrease in productive areas (Castro-Olivares et al., 2022).

Although the aforementioned bivalves, especially the introduced *R. philippinarum*, are more resistant to thermal stress than other native bivalves cultured in Galician shellfish beds, such as the clam *Venerupis corrugata* (Gmelin 1791) and the cockle *Cerastoderma edule* (Linnaeus 1758) (e.g. Domínguez et al., 2021, 2023), extreme temperatures can cause important sublethal effects, such as reduced filtration rates, impairments in reproduction and molecular processes (e.g. Domínguez et al., 2021; Vázquez et al., 2021; He et al., 2022) and even mortality after long exposure to stress or spawning events (Macho et al., 2016; Domínguez et al., 2021). For instance, sediment temperatures higher than 32 °C during four days provoked gonadal resorption and severe haemocytic invasion in *R. decussatus*, but did not affect *R. philippinarum*. However, after 3 days of exposure to an air temperature of 40 °C for 6 h during low tide and a short recovery period under ambient conditions, *R. philippinarum* showed molecular effects, with downregulation of genes from mantle tissue involved in biomineralization process (He et al., 2022).

Strategies to cope with thermal stress used by the two clam species, and by bivalves in general, involve behavioural changes such as burying deeper in the sediment, which depends on the length of siphons, or seeking thermal refuge in other habitats such as seagrass or tidal pools (Domínguez et al., 2021; Crespo et al., 2021; Román et al., 2022; Zhou et al., 2022). For example, the intertidal dwarf seagrass *Z. noltei* retains moisture and buffers temperature changes during low tides, thus improving the habitat for clams and other invertebrates (Crespo et al., 2021; Román et al., 2022). This buffering role may be mainly important in the intertidal zone, where organisms are particularly vulnerable to

environmental changes as conditions are already close to the thermal limits of the species (Jones et al., 2010). Consequently, maintenance of seagrass patches within intertidal shellfish beds could be crucial in the context of climate change as seagrass can mitigate the effects of heatwaves on bivalves (Román et al., 2022).

Bivalves, including clams and oysters, have a remarkable ability to cope with environmental stressors. They can adapt their filtration and respiration rates (Ding et al., 2020; Domínguez et al., 2021; Peruzza et al., 2023) and use biochemical defence mechanisms that increase enzyme activity, produce heat shock proteins (HSPs), accumulate free amino acids and activate antioxidant pathways (Ding et al., 2020; He et al., 2022; Masanja et al., 2022). Gene expression responses of bivalves to marine heatwaves have garnered significant attention in recent years (Xu et al., 2022; Hu et al., 2022; Jahan et al., 2022; Peruzza et al., 2023; De Marco et al., 2023). Molecular and transcriptomics studies have revealed that prolonged marine heatwaves, lasting up to 30 days, induce upregulation of HSPs, which play a critical role in mitigating heat stress and preventing the denaturation of proteins (Li et al., 2013; Masanja et al., 2022). For instance, oysters exposed to thermal stress (25 °C) for 24 days showed tissue-specific synthesis of HSPs, with HSP70 and HSP23 exhibiting more rapid upregulation in the gill than in the mantle, while HSP12A expression was specifically induced in the gills (Meistertzheim et al., 2007). In oysters, a marine heatwave lasting 30 days resulted in long-term overexpression of genes related to HSPs, including HSP70, HSP72 and HSP90 (De Marco et al., 2023). Similarly, when *R. philippinarum* was exposed to a seawater temperature of 30 °C for 6 h, HSP60 was initially upregulated and then gradually downregulated, suggesting a flexible response to heat stress and gradual acclimation (Ding et al., 2020). Understanding the genetic responses and molecular mechanisms involved in adaptation to thermal stress in bivalves is vital for comprehending the resilience of these organisms in the face of climate change-induced challenges.

The present study aimed to experimentally assess for the first time changes in gene expression during heat stress at low tide in the two aforementioned commercially-important bivalve species dwelling in two distinct habitats: bare sand and sand covered with the seagrass *Z. noltei*. We anticipated a larger number of differentially expressed genes related to heat stress in bare sand than in sand with *Z. noltei*, attributing this discrepancy to the temperature-buffering effect of the seagrass. In addition, we hypothesised that *R. philippinarum* would exhibit greater upregulation of thermal stress-related genes than *R. decussatus* with a behavioural strategy of burrowing deeper into sediment to protect against high temperatures.

2. Material and methods

2.1. Experimental set-up

The experiment was carried out in June 2022 at the Estación de Ciencias Mariñas de Toralla (CIM-ECIMAT, <https://cim.uvigo.gal/ecimat/>) belonging to the Universidade de Vigo (NW Spain). An open-air mesocosm system with four 800 L tanks (height 60 cm x width 102 cm x length 133 cm) containing two 8 L plastic boxes (height 15 cm x width 23.6 cm x length 23.6 cm), i.e. experimental units, was used. All experimental units (eight in total) were filled with sand (median particle size, 0.30 mm; previously sieved at 5 mm to remove large organisms) collected from the A Seca shellfish bed (42° 26.24'N; 8° 41.5'W). In four of the boxes, a layer of *Z. noltei* was placed on top of the sand (~90 g dw m⁻²). Therefore, the two habitat treatments, i.e. sand and sand with *Z. noltei*, simulated the intertidal conditions in shellfish beds where patches of *Z. noltei* of different extent and density coexist with bare sediment (Román et al., 2022). The boxes were immersed in running seawater of salinity 34.71 ± 0.72 and temperature of 15.33 ± 0.85 °C (mean ± SD) for one month to allow the seagrass to take root. After the one-month period, eleven individually marked juveniles of *R. decussatus* and *R. philippinarum*, both obtained from hatcheries, with shell lengths

between 15 and 18 mm, were seeded into each of the eight boxes. The juvenile clams that did not burrow within 12 h were removed and replaced with new individuals. The clams were held under the same conditions for 24 h before thermal stress was applied.

To simulate natural tidal cycles, semidiurnal tides were automatically created in each tank. During the 4 h of diurnal low tide, when the upper part of the boxes was exposed, the sediment surface in two tanks was heated to 32 °C with infrared ceramic lamps (FTE 150-W heaters, Ceramicx). The lamps were digitally controlled by sensors (Omega CN7853) which received feedback from thermocouples placed at a depth of 2 cm in the sediment in each box. Temperature of 32 °C was chosen because, in previous studies, it was stated that sediment temperatures higher than 32 °C resulted in some mortality on these species (Macho et al., 2016; Domínguez et al., 2023; 2023; Woodin et al., 2020; Vázquez et al., 2021). The elevated temperature was maintained for 3.5 h during this part of the tidal cycle. The other two tanks served as control tanks and the same tidal cycle was applied, without heating by infrared ceramic lamps, and a temperature of 20 °C was reached. The experimental conditions were repeated for four consecutive days during the simulated diurnal low tide, resembling the summer temperatures recorded in intertidal shellfish beds by iButtom data loggers (DS-1922L, Maxim Integrated, San Jose, CA) at approximately 3 cm depth in sediment during heatwaves in previous years (see Domínguez et al., 2021). Throughout the experiment, the running 80 µm-filtered seawater in the tanks was maintained at a salinity of 34.71 ± 0.72 ppm and temperature of 18 ± 0.8 °C, (mean \pm sd). The clams were fed daily in the afternoon with a maintenance diet of a microalgae mixture that included *Isochrysis galbana* (TISO), *Tetraselmis suecica*, *Chaetoceros gracilis* and *Rodomonas lens*. The amount of microalgae provided was 1% of the dry weight of the clams.

At the end of the four-day stress period, four individuals of each clam species were selected from each habitat and temperature treatment (see Supplementary Figure for a description of the experimental design). The clams were carefully dissected, and a section of approximately 2 cm was gently cut from the border of the mantle. This tissue was selected since it is known to be important in shaping responses to thermal stress in diverse bivalve species (Georgoulis et al., 2021; He et al., 2022). Each section was placed in a cryovial and immediately submerged in liquid nitrogen. The cryovials were stored at -80 °C for further analysis.

2.2. Whole transcriptome sequencing

RNA extraction was performed with the Qiagen RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's instructions. To ensure minimal enzymatic activity and RNA degradation, 2-mercaptoethanol was added to the lysis buffer. To ensure removal of all DNA, the samples were treated with DNase I during the RNA column clean-up process, in accordance with the provider (Qiagen).

The resulting RNA samples were then sent to Macrogen Inc. (South Korea) for whole transcriptome sequencing. After ribosomal RNA depletion using a Ribo-Zero ribosomal RNA removal kit (Illumina), libraries were constructed with a TruSeq RNA library kit (Illumina) and paired-end sequencing was conducted by an Illumina NovaSeq 6000 platform. The sequencing platform generated 150-base pair reads, with an insert size of 350 base pairs, with a final yield of 100 million reads per sample. On receipt of the sequencing files, quality control analysis was performed using fastqc (Andrews, 2010) to evaluate the integrity and reliability of the sequences obtained. Suboptimal libraries were excluded from further analysis due to either insufficient read counts or to low complexities. The raw FASTQ files can be accessed within the Bioproject PRJNA1013758 and SRA PRJNA1013758 (<https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1013758>).

2.3. Identification of differentially expressed transcripts and GO term enrichment analysis

The RNAseq data obtained was compared with the reference transcriptomic data available for these species in the TSA NCBI database as of March 23. For *R. philippinarum*, the reference datasets from Bioprojects PRJNA137531, PRJNA426752, PRJNA298283 and PRJNA672267 were concatenated, and any sequence redundancy was eliminated using Cdhit (Fu et al., 2012) with a similarity threshold of 0.9. Subsequently, BUSCO (Manni et al., 2021) was used to compare the completeness of the combined non-redundant dataset with the known single-copy orthologous genes specific to molluscs, as found in the OrthoDB database V11 (Kuznetsov et al., 2023). For *R. decussatus*, the reference dataset from Bioproject PRJNA170474 was used. Both transcriptomes were annotated against Swiss-Prot (e-value $\leq 1.0 \times 10^{-5}$) and the InterPro database, with Blast2GO (Götz et al., 2008) and InterProScan2 in OmicsBox – Bioinformatics Made Easy (2019), and against the Kyoto Encyclopedia of Genes and Genomes (e-value $\leq 1.0 \times 10^{-10}$), with KAAS-KEGG (Moriya et al., 2007).

Once these processes were carried out, the Salmon program (Patro et al., 2017) was used to sort, quantify, map and normalise the data against the indexed reference transcriptomes, using the Galician supercomputing centre (CESGA) informatic resources (<https://www.cesga.es/>). The resulting raw counts were normalised as transcripts per million (TPM) and imported into R (R Core Team 2021) with the tximport package (Soneson, et al., 2016), for subsequent analysis. Differential expression analysis was performed with the DESeq2 library (Love et al., 2014) to determine which genes were statistically significantly differently expressed between the different experimental groups. Differentially expressed transcripts were identified by Wald tests, which were subsequently adjusted for multiple testing using the Benjamini-Hochberg procedure. A significance threshold was set at an adjusted p-value of 0.001, coupled with a stipulated minimum fold change of 2. Subsequent visualisations, encompassing PCA and volcano plots, were generated with the ggplot2 library (Wickham et al., 2016). To assess how temperature (20 °C vs. 32 °C) and habitat type (bare sand vs. sand with *Z. noltei*) affect mantle transcriptomic profiles, we conducted species-specific two-way ANOVAs with temperature and habitat as independent factors with interaction. Transcripts exhibiting significant up- and downregulation in each of the two different habitats underwent individual Gene Ontology (GO) enrichment analysis, with a Bonferroni corrected p-value cut-off of 0.05 applied through the OmicsBox platform.

3. Results

3.1. Reference transcriptome validation and sample quality control

A reference transcriptome for both *Ruditapes* species was constructed by concatenation of available transcriptome data, followed by the removal of redundancy by means of CD-hit-EST. This approach reduced the initial dataset of 676,688 transcripts to 380,929 in the case of *R. philippinarum*, while retaining 69.9% completeness based on BUSCO assessment (Supplementary Table 1). In the case of *R. decussatus*, the reference dataset generated comprised 39,414 transcripts and demonstrated a completeness of 61.2% (Supplementary Table 1). Importantly, both transcriptomes exhibited equivalent completeness values, thus validating their use for comparative RNAseq analysis between the two species.

3.2. Transcriptomic responses to thermal stress and habitat influence

Confirming our initial hypotheses, the results highlighted distinct patterns of gene expression between species, as well as some effect of the habitat on the transcriptomic response to heat conditions. The transcriptomic profiles of *R. philippinarum* revealed a profound impact of the

temperature treatments on this species. The clear separation of the samples exposed to 32 °C in bare sand from the remaining samples across the first component of the PCA (Fig. 1a) was particularly noteworthy, implying that the transcriptomic profiles of *R. philippinarum* exposed at 32 °C in the *Z. noltei* habitat were actually more similar to the experimental samples exposed at 20 °C in both habitats. This finding was supported by the results of a two-way ANOVA analysis, which revealed a marginally significant effect of temperature depending on the habitat (Table 1). However, in the case of *R. decussatus*, there were no significant alterations in the overall gene expression profiles across the experimental conditions, as supported by the results of the two-way ANOVA analysis of the first PCA component (Fig. 1b). Indeed, the transcriptomic analysis revealed a larger overall number of differentially expressed genes (DEGs) in *R. philippinarum* samples than in their *R. decussatus* counterparts. Volcano plot representations further supported the observation that *R. philippinarum* (Supplementary Tables 2–3, Fig. 1 c-d) exhibited significantly more DEGs in response to thermal stress ($p < 0.01$) than *R. decussatus* (Supplementary Tables 4–5, Fig. 1 e-f). Altogether these observations strongly suggested that *R. decussatus* exhibited less transcriptomic plasticity in response to temperature stress under the specific experimental conditions tested and that the presence of the seagrass *Z. noltei* could at least partly ameliorate the impact of heat on the transcriptome of *R. philippinarum*.

3.3. GO enrichment and KEGG-KAAS analysis

Enrichment analyses for GoTerms were only conducted for *R. philippinarum*, as this species exhibited the largest number of DEGs at the two temperatures tested (Fig. 2; Supplementary Table 6). The bare sand (Fig. 2a) habitat exhibited significant enrichment of GoTerms associated with the cellular response to heat, including heat shock-mediated polytene chromosome puffing (GO:0035080) and cellular heat acclimation (GO:0070370), indicating activation of stress response pathways. Moreover, GoTerms related to regulation of protein stability and chaperone activity, such as regulation of protein complex stability (GO:0061635) and positive regulation of protein refolding (GO:1904592), were also prominent, suggesting upregulation of heat shock protein activity. Stress-related GoTerms, encompassing response to cold (GO:0009409), xenobiotic stimulus (GO:0009410), starvation (GO:0042594) and other factors, were enriched in this habitat, as direct responses to thermal stress and related stressors. Additionally, autophagy (GO:0061740, GO:0061738), apoptosis (GO:0010667) and meiotic-related terms (GO:0032570, GO:0044849) were also enriched, indicating diverse cellular responses to thermal stress in this habitat.

By contrast, the sand with *Z. noltei* habitat exhibited more limited enrichment in stress-related GoTerms (Fig. 2b, Supplementary Table 7). Only a few GoTerms were directly associated with heat (GO:1904592 and GO:0035080) or protein refolding (GO:0051085, GO:1904764, GO:1904592, GO:0061635). This observation suggests that the impact of the heatwave on *R. philippinarum* in this habitat was weaker than in the same clams in the bare sand habitat. Additionally, GoTerms related to ATP metabolism (GO:0046034), mRNA editing (GO:0048026) and meiosis (GO:0044849) were also identified. The reduced enrichment of stress-related GoTerms in this habitat relative to that of *R. philippinarum* clams held in bare sand indicates potential mitigation of *Z. noltei* to heat stress effects in this species.

3.4. Genes involved in heatwave responses

The cellular response of *R. philippinarum* to heat stress was characterised by pronounced activation of Heat Shock Proteins (HSPs) (Fig. 3a). Specifically, *HSPA5* (GIVV01033789.1) displayed gene activation in response to heat specifically in the bare sand habitat. Similarly, *HSPA2* (GIVU01000444.1), also known as *HSP70-2*, and *CRYAB* (GHAV01309706.1), also known as *HSPB5*, exhibited minimal expression at 20 °C, but significantly higher levels at elevated temperature in

both habitats, albeit to a lesser extent in the sand with *Z. noltei* habitat. Moreover, in the sand with *Z. noltei* habitat was gene activation was enhanced for transcripts such as *CTSC* (GIVR01012960.1), encoding for a chaperone, as well as two unnamed proteins related to chromosome puffing in response to heat (GHAV01140329.1 and GAEH01003586.1).

In the context of mitochondrial metabolic pathways, several genes were identified in *R. philippinarum* as being differentially expressed in response to heat stress (Fig. 3a). Notably, transcripts encoding subunits of the Mitochondrial Complex I (MtCI), *NDUFS8* (GIVV01004452.1) and *NDUFA12* (GIVS01032289.1 and GHAV01192566.1) were activated by heat stress in the bare sand habitat, while in the presence of *Z. noltei*, these genes were not significantly altered. By contrast, *ND4* (GIVW01008634.1) and some components of the Mitochondrial Complex IV (MtCIV), present downstream in the mitochondrial oxidative phosphorylation pathway, such as *COX1* (GIVW01036588.1) and *COX3* (GAEH01001862.1), were downregulated in both habitats, although only significantly for the habitat with *Z. noltei*. Similarly, *TOM7* (GAEH01005375.1), involved in protein import towards the mitochondria, was selectively downregulated in response to heat stress in the habitat with *Z. noltei*.

The response of *R. philippinarum* to thermal stress also revealed that the habitats showed distinct patterns of expression of various transcripts associated with apoptosis (Fig. 3a). Particularly, genes encoding apoptosis inhibitor proteins (IAPs) showed contrasting responses: *BIRC7* (GHAV01215382.1) was upregulated in both habitats, while *BIRC2* (GIVW01016367.1) and *BIRC3* (GGKI01001272.1, also known as *IAP2*) exhibited selective upregulation in the bare sand habitat. Other IAPs, *BTF3L4* (GAEH01002639.1) and *TAX1BP1* (GIVW01030441.1), were downregulated in response to elevated temperature in the habitat with *Z. noltei*. Additionally, *SRPX* (GIVR01020576.1), involved in the positive regulation of autophagy and extrinsic apoptosis, was specifically downregulated in the bare sand habitat. Other transcripts with cellular functions either tangentially or not related *a priori* to heat stress response, such as cytoskeleton dynamics (*TPPP3*: GIVT01033117.1, *TUBA1B*: GGKI01103471.1, *TAGLN*: GIVT01042087.1 and *MUC6*: GIVR01000493.1), neurogenesis (*CIQL*: GIVT01042247.1) or activation of signalling pathways (*TMPRSS9*: GHAV01315105.1) were also upregulated in response to heat in the bare sand habitat, while *KLHL17K*: GIVW01035949.1, also involved in the regulation of the cytoskeleton, protein degradation and signalling pathways were found downregulated in both habitats.

Despite the limited set of DEGs detected in *R. decussatus*, some transcripts associated with the cellular response to heat were noted (Fig. 3b). These genes included those encoding three heat shock proteins, two of which corresponded to Hsp20 (GFXP01008387.1, GFXP01029631.1 and GFXP01011967.1), an unnamed chaperone involved in protein refolding (GFXP01021635.1) and, related to proteolysis (GFXP01024765.1) or aminoacidic transport (GFXP01017551.1). *DAZAP2* (GFXP01027628.1), was also overexpressed in both habitats, although its association with heat stress remains elusive to our current understanding. Notably, all of these genes exhibited significant upregulation in response to heat stress in both habitats, probably playing pivotal roles in facilitating acclimation to heat in this species. While the overexpression of these genes demonstrated some effect of thermal stress on the transcriptome of *R. decussatus*, no significant differences were observed between the two habitats.

4. Discussion

In intertidal shellfish beds, clams inhabit an ever-changing environment characterised by the cyclical transition between aquatic and terrestrial states due to tidal fluctuations. Consequently, they must adapt to a broad spectrum of environmental variables, including salinity, temperature, food availability and hypoxia (Li et al., 2013). Most notably, they experience substantial increases in sediment temperatures during emersion, particularly during summer when elevated

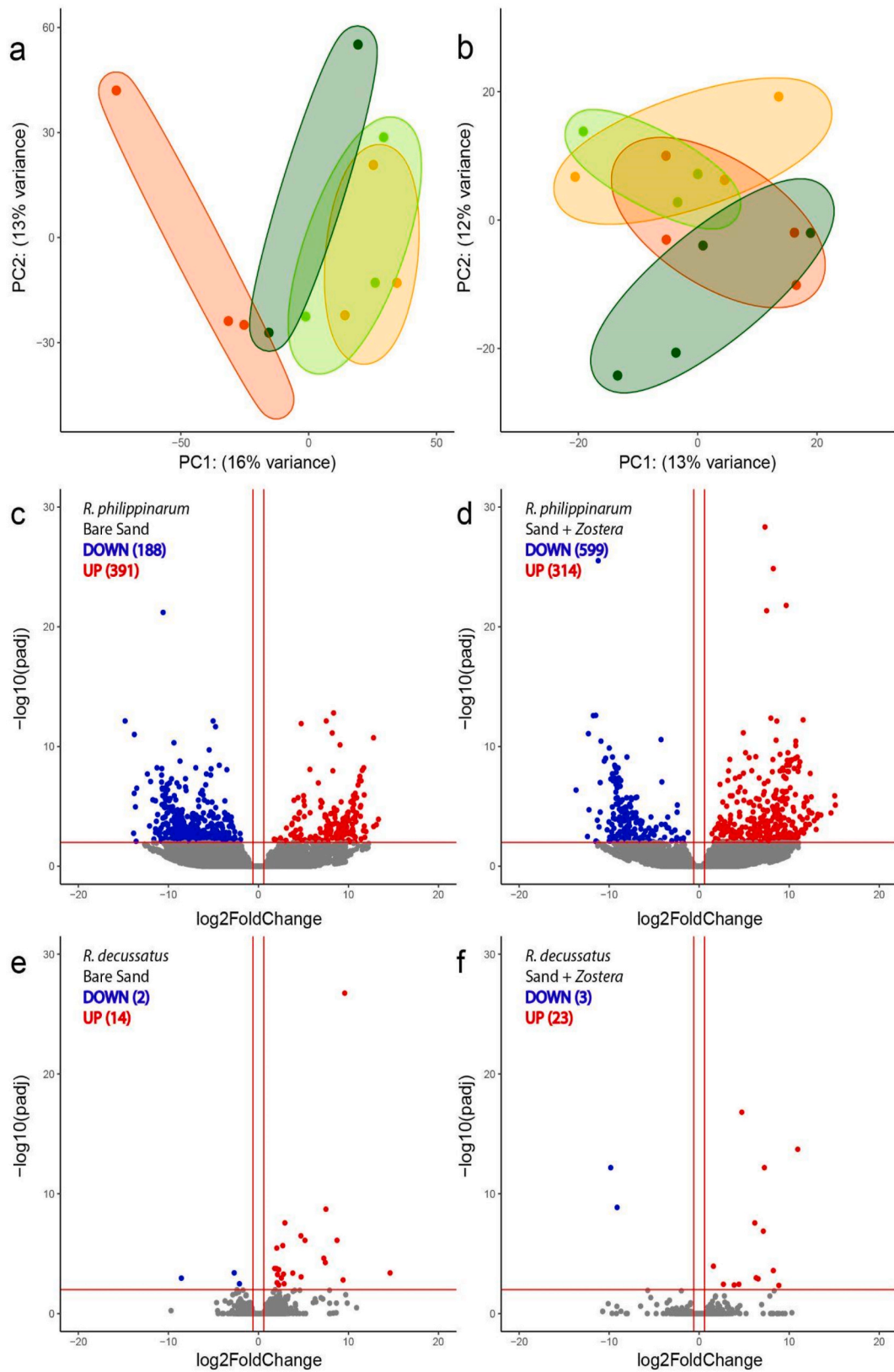


Fig. 1. Impact of temperature on the transcriptomic profiles of the mantle of both clam species in the different habitats. Principal Component Analysis (PCA) plots show the transcriptomic profiles of *R. philippinarum* (a) and *R. decussatus* (b) under varying temperature and habitat conditions. Light green: sand with *Z. noltei* at 20 °C; green: sand with *Z. noltei* at 32 °C; light orange: sand at 20 °C; orange: sand at 32 °C. Volcano plots illustrate differentially expressed genes in response to heat stress (20 °C vs 32 °C) for each *Ruditapes* species within the respective habitats. Significant DEGs are highlighted: red indicates overexpressed genes (UP, $p < 0.001$ and fold change > 2) and in blue indicates downregulated genes (DOWN, $p < 0.001$ and fold change < -2). Unaltered genes are depicted in grey. Regardless of the habitat, *R. philippinarum* exhibited a greater number of heat-induced DEGs under both habitat types (a: bare sand, b sand with *Z. noltei*) than *R. decussatus* (c: bare sand, d sand with *Z. noltei*).

Table 1

Analysis of variance of the first coordinate of the principal component analysis based on gene expression data obtained from *R. philippinarum* and *R. decussatus* under different experimental conditions. The design includes Temperature (control- 20°C- vs 32 °C) and Habitat type (bare sand vs sand with *Z. noltei*), as fixed orthogonal factors, and their interaction.

Dof	<i>R. philippinarum</i>					
	Sum Sq.	Mean Sq.	F	p value	Pr (>F)	
Temperature	1	5625	5625	13.583	0.0078	**
Habitat	1	711	711	1.716	0.2316	
Temperature*Habitat	1	1919	1919	4.635	0.0683	.
Residuals	7	2899	414			
<i>R. decussatus</i>						
Temperature	1	143.8	143.78	0.743	0.409	
Habitat	1	138.2	138.19	0.714	0.418	
Temperature*Habitat	1	2.2	2.17	0.011	0.918	
Residuals	7	1935.1	193.51			

Statistical significance: 0 '****' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '.' 1.

temperatures prevail (Domínguez et al., 2021). This transition induces distinct behavioural responses, including valve closure, which despite preserving moisture and shield against desiccation and thermal stress, also leads to the accumulation of metabolites, oxidative stress and anaerobic conditions. Clams may also exhibit an alternative adaptive strategy by seeking out thermal refuges, including deeper sediment or habitats such as *Z. noltei* patches (Macho et al., 2016; Domínguez et al., 2021; Román et al., 2022). This approach becomes essential, as valve closure alone fails to completely shield clams from thermal stress, primarily due to the high thermal diffusion rate in the aragonite (Woodin et al., 2020) – the principal form of calcium carbonate (CaCO₃) found in the shells of *Ruditapes* species (Mu et al., 2018).

Complementary to these behavioural strategies, a complex cascade of molecular mechanisms occurs at the cellular level, which is equally instrumental in coping with heat stress in these species (Meisterzheim et al., 2007; Li et al., 2013; Masanja et al., 2022). This includes a spectrum of differentially expressed genes, resulting in variations in protein synthesis that impose additional energetic costs to the organism (Ferreira-Rodríguez et al., 2018). The interplay between behavioural adaptation, habitat, molecular responses and the energy required to activate these mechanisms, as it was demonstrated in this paper, highlights the dynamic balance between the diverse strategies that the organisms may develop to cope with thermal stress. Here, we sought to unravel for the first time the intricate interplay between the responses to heat stress in two abundant, commercially important species of *Ruditapes*.

The data presented herein provide compelling evidence indicating distinct adjustments of gene expression in response to heat stress in both *R. decussatus* and *R. philippinarum*. Notably, *R. decussatus* exhibited fewer differentially expressed genes (DEGs), suggesting potentially greater resistance to heat stress than in *R. philippinarum*. These findings are consistent with our initial hypothesis and with previous findings indicating the superior thermal tolerance of *R. decussatus* than of *R. philippinarum* (Velez et al., 2017). Nevertheless, we must emphasise that our experimental set-up was carefully designed to replicate the effects of heat shock stress on the mantle transcriptomic profiles of both clam species in their natural environmental conditions. This was accomplished by mimicking high and low temperatures during low tides in different shellfish bed habitats: bare sand and patches of sand covered with the seagrass *Z. noltei*. By contrast, some previous studies were conducted either without sediment (Nie et al., 2017; Velez et al., 2017; Bae et al., 2021; He et al., 2022; Zhang et al., 2022) or with an arguably insufficient sediment column height, greatly deviating from natural conditions (but see Crespo et al., 2021). Another notable divergence from the prevailing norm in prior studies assessing heat stress in bivalves

(e.g. Zhang et al., 2022) is the incorporation of tidal oscillations alternating periods of emersion and immersion in our experimental design. This was pivotal to capturing the full spectrum of stress responses in organisms inhabiting intertidal areas. Our investigation also represents a pioneering step towards accounting for the influence of the habitat context in modulating heat stress responses on these bivalve species.

Under these circumstances, it is reasonable to conclude that the observed differences in transcriptomic responses of the two species can be attributed to their distinct behavioural strategies. Thus, *R. decussatus* displays a remarkable capacity for rapid, deep burrowing in sediment, with adult specimens reaching depths greater than 13 cm (Macho et al., 2016; Lopes et al., 2018; Domínguez et al., 2021), which provides them a mechanism to withstand heat due to the thermal diffusion of sand (Macho et al., 2016; Domínguez et al., 2021; Román et al., 2022) without the need for extensive transcriptomic modifications. On the other hand, *R. philippinarum* displays less proficient burrowing abilities, reaching depths of around 8 cm (Lee, 1996; Macho et al., 2016; Domínguez et al., 2021), making this species more vulnerable to the impact of heat stress (Macho et al., 2016). Consequently, *R. philippinarum* must rely on significant adaptations in its metabolic pathways to effectively withstand and manage stress caused by escalating temperatures (Macho et al., 2016; Domínguez et al., 2021).

Temperature tolerance is a multifaceted trait influenced by the interplay between multiple genes, leading to a complex pattern of gene expression during stress events (Jahan et al., 2022). In response to heat stress, both *R. philippinarum* and *R. decussatus* exhibited upregulation of genes encoding heat shock proteins (HSPs) and chaperone proteins, including HSPA5 and HSPA2. However, in *R. decussatus* the upregulation was more subtle, consistent with previous findings (Velez et al., 2017) and in line with our initial hypothesis. These proteins play crucial roles in repairing nuclear proteins and safeguarding cells from the adverse effects of environmental stress (Yao and Somero 2012; Ding et al., 2018), and their expression levels are directly linked to thermal tolerance in different clam species, making them valuable biomarkers for assessing stress (Nie et al., 2017). The metabolic response to heat stress comes with substantial energy costs, especially for the synthesis of HSPs (Zhang and Dong 2021). The energy expenditure associated with protein synthesis emphasizes the energetic trade-offs involved in this process and defines the behavioural strategy of the organisms. *R. decussatus* circumvents the need to allocate resources to gene expression or protein synthesis, choosing instead to invest energy in the energetically costly process of burrowing more deeply, estimated to be as much as 7% of the total reserves of some bivalves (Haider et al., 2018).

The response of genes involved in mitochondrial oxidative phosphorylation to thermal stress in *R. philippinarum* suggests a complex strategy to ensure cellular survival and maintain homeostasis. Upregulation of genes encoding subunits of the Mitochondrial Complex I (MtCI) probably enhances electron transfer efficiency, leading to reduced generation of reactive oxygen species (ROS) and consequent mitigation of oxidative stress provoked by high temperatures (de Almeida et al., 2007; Georgoulis et al., 2021). The observed response may also be attributed to temporary upregulation of the aerobic metabolism in response to escalating environmental demands, including rising temperatures (Thomas and Bacher 2018), as previously observed for *R. philippinarum* (Zhang et al., 2023) and *Mytilus galloprovincialis* Lamarck 1819; Anestis et al., (2010). Conversely, the downregulation of genes downstream in the mitochondrial chain in *R. decussatus* may suggest activation of an alternative oxidase pathway (AOX). AOX has been proposed to support survival in anoxic conditions in some bivalves (Yusseppone et al., 2018), suggesting that this modulation may contribute to the acclimation of clams to diverse environmental conditions. Indeed, prior data confirm downregulation of genes associated with mitochondrial metabolism, such as COX1 and 16S, probably as a consequence of these species using a behavioural strategy of valve closure to cope with increasing temperatures (Velez et al., 2017; Bae et al., 2021). Pronounced closure of valves would constrict gas

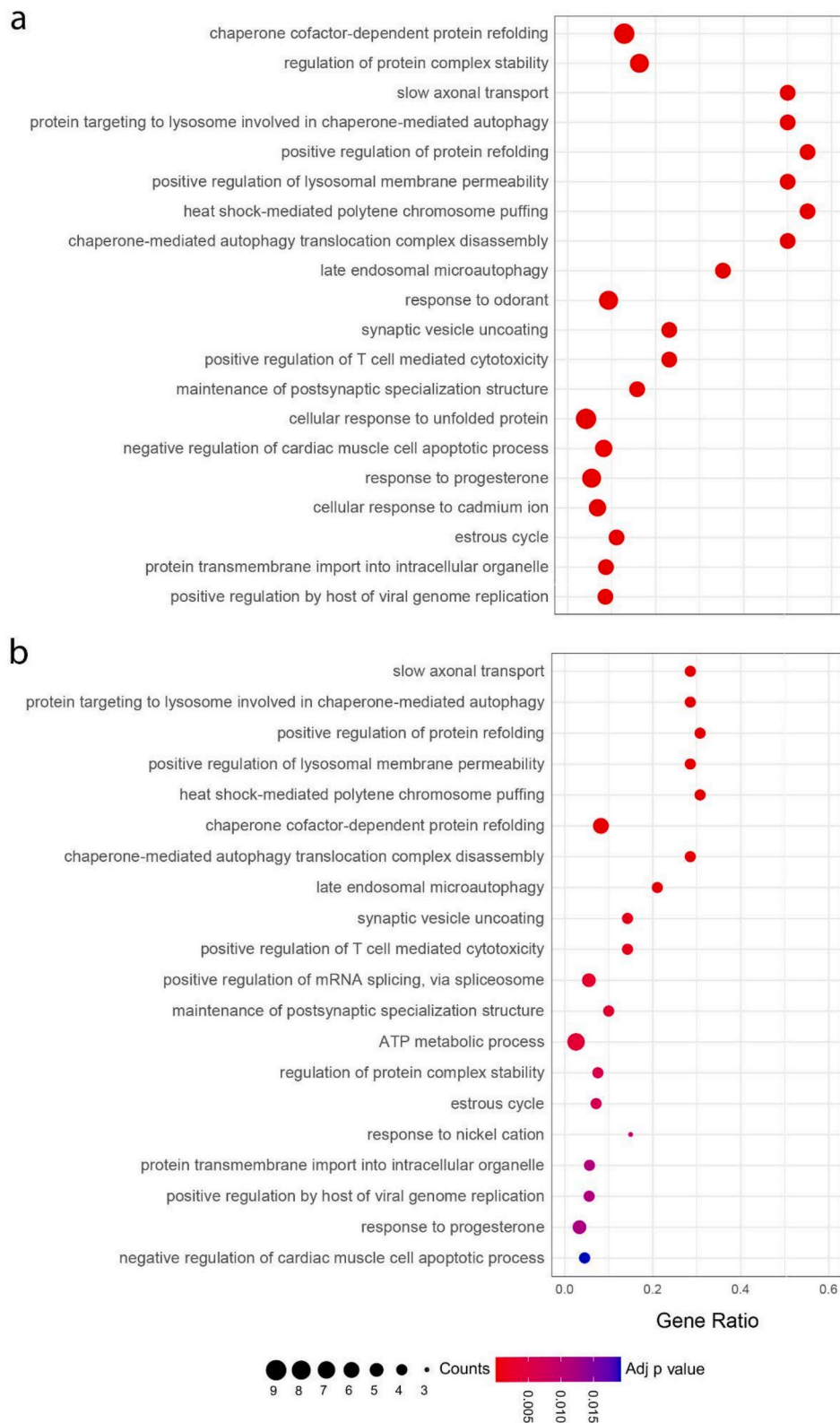


Fig. 2. Dot plots depicting the top 20 significantly enriched biological processes GO terms (FDR <0.05) of the heat-stress differentially expressed genes (20 °C vs 32 °C) identified using Omicsbox in *R. philippinarum* at the two different habitats: (a) bare sand and (b) sand with *Z. noltei*. The color gradient corresponds to adjusted P-values, while variations in bubble size align with the enrichment factor.

exchange, thereby constraining aerobic metabolism and promoting the activation of AOX pathways.

Previous studies have shown that bivalves exposed to high temperatures, along with other stressors, experience significant dysregulation

of autophagocytosis and apoptotic genes (Ghaffari et al., 2019; Song et al., 2021; Jahan et al., 2022). In this regard, IAPs are actively involved in stress responses induced by various stressors, contributing to the environmental resilience of bivalves (Song et al., 2021). It is known that

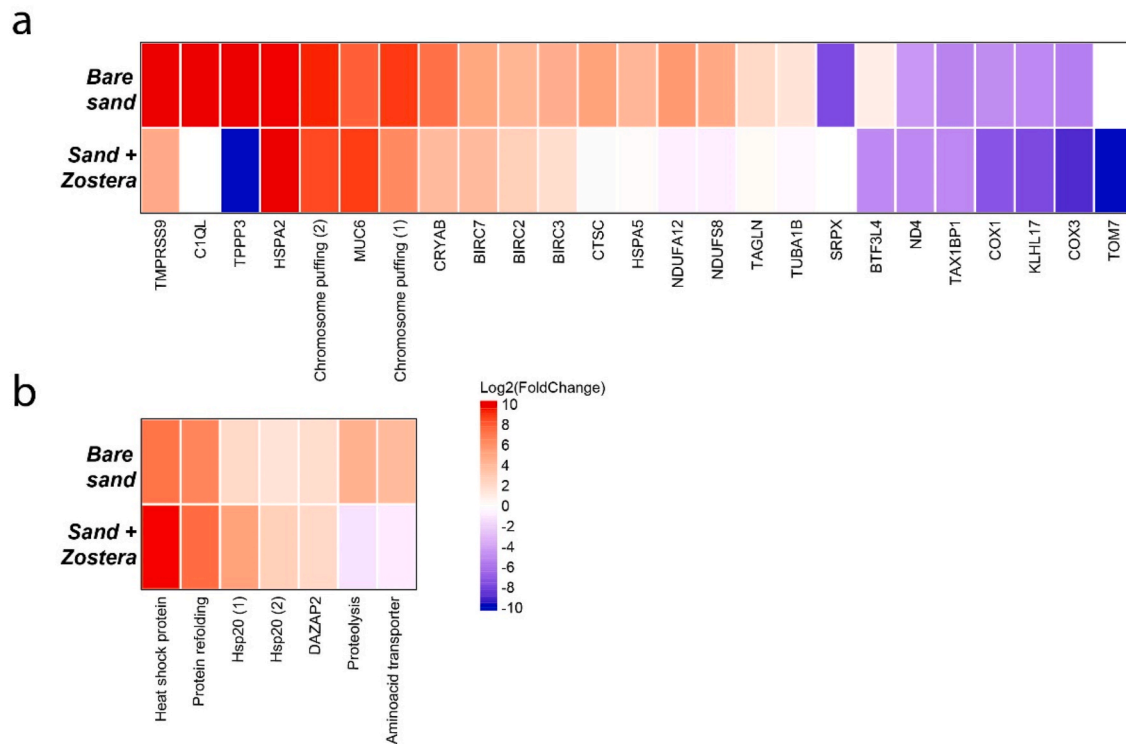


Fig. 3. Heatmap comparisons of differentially expressed genes in response to heat stress (20 °C vs 32 °C) in *R. philippinarum* (a) and *R. decussatus* clams in the two habitats, bare sand and sand with *Z. noltei*, capturing both overexpressed and repressed genes.

IAP genes in bivalves have undergone genome expansion, intricately linked to a refined specialisation and differential expression both in terms of organ or tissue specificity and their distinct sensitivity to various environmental stressors (Song et al., 2021). Furthermore, our results are consistent with previous findings, revealing differential expression of genes related to cytoskeleton dynamics, including GSN, DYNLRB, TMP1 and DYNLL, in response to heat stress. These results suggest the existence of common mechanisms among bivalves to mitigate heat stress, consistent with the findings of Hu et al. (2022).

Notably, the presence of the seagrass *Z. noltei* in the habitat appears to play a role in mitigating the impact of heat stress on the transcriptome of *R. philippinarum*. This remarkable finding highlights the intricate relationship between environmental factors and the transcriptomic response of these clams to thermal challenges, consistent with previous findings (Román et al., 2022, 2023). In this regard, the presence of seagrass may confer direct benefits to the bivalve in terms of energy conservation in the synthesis of specific proteins or gene expression. This energy saved could be reallocated towards other crucial biological processes, such as growth and reproduction, which that are energetically costly (Vázquez et al., 2021). This may be particularly relevant for bivalve species, considering that heatwaves coincide with their spawning season, which usually coincides with massive mortality events observed in these species (Aranguren et al., 2014). Indeed, seagrass constitutes a formidable buffer against environmental stressors and heightened mortality rates in bivalves confronting diverse challenges such as decreased salinity and exceptional temperature fluctuations (de Fouw et al., 2016; Gagnon et al., 2020; Cardini et al., 2022, Herrera et al., 2023). By understanding the role of seagrass in ameliorating the effects of heat stress, shellfishers could improve shellfish beds management to enhance the resilience of clam species to increasingly prevalent and severe heatwaves (Guimarães et al., 2012; Bas Ventín et al., 2015). Approaches such as maintaining zones of bare sediment interspersed with areas of *Z. noltei* cover could establish the foundation for shellfish production that is both resilient and sustainable, thus effectively addressing the increasing frequency of heatwaves caused by climate

change (Román et al., 2022).

5. Conclusion

Our study findings highlight the impact of heatwaves on *Ruditapes* species, elucidating the species-specific responses that lead to modified gene expression as well as differences in transcriptomic responses that can be attributed to their distinct behavioural strategies. *Ruditapes philippinarum* exhibited robust upregulation of numerous genes and significant pathway modifications, showcasing its ability to cope with heat stress, particularly in the absence of *Z. noltei*, suggesting a potential role of this phanerogam in mitigating thermal stress. Conversely, *R. decussatus* displayed less variation in gene expression than in its basal state, which may be attributed to the remarkable ability of this species to burrow to a depth of 15 cm where the sediment is cooler (owing to thermal diffusion), thus enabling the species to withstand heatwaves. Our findings shed light on the diverse adaptation strategies used by these bivalves in response to thermal challenges, providing valuable insights into their thermal tolerance mechanisms. Moreover, in the light of our results, we propose a new shellfish management strategy, namely maintaining areas of *Z. noltei* in the intertidal shellfish beds to improve the resilience of clams to heatwaves. Further investigations are warranted to improve our understanding of the underlying molecular mechanisms and environmental interactions that shape the responses of these bivalve species to heat stress.

Funding source

This research was supported by the Spanish Ministerio de Ciencia e Innovación/Agencia Estatal de Investigación (10.13039/501100011033) for the ZEUS project (grant RTI2018-095583-B-I00) and by the Autonomous government Xunta de Galicia-FEDER (project ED431C 2021/42 and ED431C 2020/05): "ERDF A way of making Europe". DGS is supported by a postdoctoral contract from Xunta de Galicia (ED481D 2022/001).

CRediT authorship contribution statement

Daniel García-Souto: Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Víctor Martínez-Marino:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Paloma Morán:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Celia Olabarria:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Elsa Vázquez:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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.

Data availability

Other (please explain: e.g. ‘I have shared the link to my data as an attachment’).

Acknowledgements

This research was supported by the Spanish Ministerio de Ciencia e Innovación/Agencia Estatal de Investigación (10.13039/501100011033) for the ZEUS project (grant RTI2018-095583-B-I00) and by the Autonomous government Xunta de Galicia-FEDER (project ED431C 2021/42 and ED431C 2020/05): ‘ERDF A way of making Europe’. DGS is supported by a postdoctoral contract from Xunta de Galicia (ED481D 2022/001). We thank S. Woodin, D. Wethey, M. Román, S. Román, J. Troncoso, E. Pérez and M. Pérez for valuable assistance with laboratory work. We are also very grateful to anonymous reviewers and the Associate Editor for their constructive comments, which helped us to improve the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jtherbio.2023.103776>.

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