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Environmental transcriptomics under heat stress: Can environmental RNA reveal changes in gene expression of aquatic organisms?

Robert M. Hechler[1] | Matthew C. Yates[2] | Frédéric J. J. Chain[3] | Melania E. Cristescu[1]

[1]Department of Biology, McGill University, Montréal, Québec, Canada

[2]Département des Sciences Biologiques, Université du Québec à Montréal, Montréal, Québec, Canada

[3]Department of Biological Sciences, University of Massachusetts Lowell, Lowell, Massachusetts, USA

Correspondence
Robert M. Hechler, Department of Biology, McGill University, Montréal, QC, H3A 1B1, Canada.
Email: robert.hechler@mail.utoronto.ca

Present address
Robert M. Hechler, Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, Ontario, Canada

Matthew C. Yates, Great Lakes Institute for Environmental Research, University of Windsor, Windsor, Ontario, Canada

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Abstract

To safeguard biodiversity in a changing climate, taxonomic information about species turnover and insights into the health of organisms are required. Environmental DNA approaches are increasingly used for species identification, but cannot provide functional insights. Transcriptomic methods reveal the physiological states of macroorganisms, but are currently species-specific and require tissue sampling or animal sacrifice, making community-wide assessments challenging. Here, we test whether broad functional information (expression level of the transcribed genes) can be harnessed from environmental RNA (eRNA), which includes extra-organismal RNA from macroorganisms along with whole microorganisms. We exposed *Daphnia pulex* as well as phytoplankton prey and microorganism colonizers to control (20°C) and heat stress (28°C) conditions for 7 days. We sequenced eRNA from tank water (after complete removal of *Daphnia*) as well as RNA from *Daphnia* tissue, enabling comparisons of extra-organismal and organismal RNA-based gene expression profiles. Both RNA types detected similar heat stress responses of *Daphnia*. Using eRNA, we identified 32 *Daphnia* genes to be differentially expressed following heat stress. Of these, 17 were also differentially expressed and exhibited similar levels of relative expression in organismal RNA. In addition to the extra-organismal *Daphnia* response, eRNA detected community-wide heat stress responses consisting of distinct functional profiles and 121 differentially expressed genes across eight taxa. Our study demonstrates that environmental transcriptomics based on extra-organismal eRNA can noninvasively reveal gene expression responses of macroorganisms following environmental changes, with broad potential implications for the biomonitoring of health across the trophic chain.

KEYWORDS
biological monitoring, biomonitoring, environmental DNA (eDNA), environmental RNA (eRNA), environmental transcriptomics, extra-organismal RNA, gene expression

1 | INTRODUCTION

In an era of ecological crises, monitoring the presence and health of organisms within an ecosystem is essential for the preservation of biodiversity. Environmental DNA is routinely used for biomonitoring, but such applications are limited to species detection (Cristescu & Hebert, 2018; Deiner et al., 2017). The RNA molecule provides an additional layer of functional information, as transcribed genes reflect an organism's physiological status (Huang et al., 2002). Thus, transcriptomics is used to gain insights into the physiological status of organisms and subsequently infer their health (Connon et al., 2018; Jeffries et al., 2021), such as salmon exhibiting heat and osmotic stress and active disease states (Akbarzadeh et al., 2021; Houde et al., 2019; Miller et al., 2017). However, such surveys are species-specific and dependent on sampling organismal RNA (oRNA) directly, which requires tissue collection or animal sacrifice (Akbarzadeh et al., 2021; Baillon et al., 2015; Gleason & Burton, 2015; Houde et al., 2019; Miller et al., 2017). Noninvasive environmental RNA (eRNA) approaches could overcome these limitations by providing insights into the health of populations and communities (Amarasiri et al., 2021; Cristescu, 2019; Veilleux et al., 2021; Yates et al., 2021). However, using eRNA to inform on the health of macroorganisms has only been theorized (see Yates et al., 2021) and remains to be empirically tested.

We broadly define eRNA as RNA extracted from the environment, including extra-organismal RNA released by macroorganisms as well as whole microorganisms. Microbiologists have long extracted RNA from bulk samples of microorganisms to profile gene expression in microbial communities, referred to as metatranscriptomics (Frias-Lopez et al., 2008; Gilbert et al., 2008; Poretsky et al., 2005). Despite more than two decades of research, metatranscriptomics remains limited to studying the gene expression of microorganisms captured in samples rich in microbial oRNA. The general assumption has been that the extra-organismal RNA released by macroorganisms into the environment is labile and degrades too rapidly to be reliably detected or quantified (but see Cristescu, 2019). However, several laboratory experiments demonstrate that the RNA shed by macroorganisms into their surrounding environment can be successfully extracted and detected for up to 13 (Wood et al., 2020), 72 (Marshall et al., 2021) and 57 (Kagzi et al., 2022) hours. Jo et al. (2022) highlighted the importance of temperature on eRNA decay rates as they found that half-life times of two zebrafish eRNA genes are higher in 20°C (26 and 42 h) compared to 30°C (16 and 15 h), suggesting that eRNA might degrade faster at higher temperature. eRNA has also been extracted and metabarcoded from freshwater and marine systems to reveal the composition of metabolically active biotic communities (Littlefair et al., 2022; Miyata et al., 2021; von Ammon et al., 2019). Despite emerging evidence supporting the extractability of eRNA, extra-organismal eRNA had not been previously used to analyse macroeukaryotic stress responses. This type of 'environmental transcriptomics' differs from the rich metatranscriptomics literature in that it targets macroorganisms using extra-organismal eRNA instead of oRNA derived from microorganisms.

In aquatic systems, environmental conditions modulate biodiversity and are reflected in changes of metatranscriptomes (Frias-Lopez et al., 2008; Salazar et al., 2019; Sunagawa et al., 2015; Sunday et al., 2011). Temperature is a key abiotic factor that influences the physiology and fitness of ectotherms due to the relationship between body and environmental temperatures (Huey & Berrigan, 2001; Rodgers, 2021; Schulte, 2015). Rapid increases in temperature, such as heatwaves, can exceed the thermal limits of aquatic ectotherms and exponentially increase metabolic rates, resulting in decreases in fitness and survival (Harley et al., 2006; Reid et al., 2019; Rodgers, 2021; Schulte, 2015). Transcriptomics is widely used to study the heat stress response of many taxa, including fish (Akbarzadeh et al., 2018; Houde et al., 2019; Narum & Campbell, 2015; Veilleux et al., 2015, 2018), coral holobionts (Savary et al., 2021; Voolstra et al., 2021), molluscs (Chen et al., 2019; Gleason & Burton, 2015) and copepods (Kelly et al., 2017; Semmouri et al., 2019). *Daphnia pulex*, a key bioindicator species, exhibits widespread downregulation of genes involved in metabolic processes under heat stress (Becker et al., 2018; Yampolsky et al., 2014). Clearly, gene expression surveys can provide species-specific functional insights important for conserving aquatic biodiversity (Bozinovic & Pörtner, 2015; Evans & Hofmann, 2012; Wikelski & Cooke, 2006). However, a species-by-species analysis based on oRNA cannot capture community-wide changes without extensive efforts. Mapping eRNA reads to the reference genomes of multiple target species and using existing metatranscriptomic pipelines could potentially enable environmental transcriptomics to reveal gene expression responses across the trophic chain, including macroorganisms.

We constructed simple mock freshwater communities containing *D. pulex*, as well as three phytoplankton species and opportunistic microorganisms that colonized the artificial lake water. The communities were exposed to control (20°C) and near-lethal *Daphnia* heat stress (28°C) conditions for 7 days. To enable comparisons between eRNA- and oRNA-based gene expression profiles, we sequenced eRNA from tank water (after complete *Daphnia* removal) and oRNA from *Daphnia* tissue. We conducted differential gene expression analyses between the control and heat stress conditions and compared the *Daphnia* functional stress response identified in eRNA and oRNA. In addition to the extra-organismal *Daphnia* analysis, we used eRNA to investigate the community-wide heat stress response. We hypothesized that (1) eRNA captures transcriptional responses to heat stress and (2) eRNA contains a subset of the differentially expressed *Daphnia* genes observed in oRNA after exposure to heat stress. Based on our findings, we discuss the potential of eRNA-based transcriptomics for the biological monitoring of macroorganisms and ecological communities and also provide recommendations to overcome potential limitations.

2 | MATERIALS AND METHODS

2.1 | Experimental design

We seeded four tanks (biological replicates) containing 8 L artificial lake water (Celis-Salgado et al., 2008) with a starting population of 50 parthenogenetic females of *Daphnia pulex* (clone from Illinois, USA; 40.24, −87.78). *Daphnia* populations were kept in controlled chambers for 45 days at control growing conditions: 20°C, relative humidity of 50% and a photoperiod of 16:8 h light: dark. *Daphnia* were fed 1.5 mL algae (*Ankistrodesmus falcatus*, *Scenedesmus quadricauda* and *Pseudokirchneriella subcapitata* at concentrations of 5,517,778 cells/mL, 22,013,332 cells/mL and 25,133,333 cells/mL, respectively) diluted in 200 mL artificial lake water, twice per week. After 45 days, the *Daphnia* populations in all tanks reached a minimum of 500 individuals and we began the experiment by transferring tanks to controlled experimental chambers pre-set to the respective temperatures (20°C and 28°C). These simple mock freshwater communities, consisting of *D. pulex*, *A. falcatus*, *S. quadricauda*, *P. subcapitata* and microorganisms that colonized the artificial lake water were exposed to 20°C (control) and 28°C (neat-lethal stress for *Daphnia*) temperatures for 7 days to mimic natural heatwave conditions and allow for eRNA accumulation in tank water.

2.2 | Sample collection

We collected four eRNA and oRNA technical replicates per tank. We submerged all experimental and sampling equipment in a 20% bleach solution for 15 min and rinsed thoroughly five times with distilled water prior to use. We stirred the tanks for 60 s and filtered 500 mL of water through a 60 μm mesh. This filtering step removed all *Daphnia*, whose body size ranges between 0.6 (first instar juvenile) and 3 mm (adult; Lynch, 1983; Tollrian, 1995), as well as ephippia that are 0.25–1 mm long (Bernatowicz et al., 2018; Davison, 1969), ensuring that extra-organismal *Daphnia* eRNA was captured in our filtered water samples. To capture eRNA, the *Daphnia*-free water was then filtered through a 0.7 μm glass microfiber filter. Each filter was cut in half and placed in individual 1.5 mL microcentrifuge tubes containing 370 μL RLT buffer (Qiagen) and 3.7 μL β-mercaptoethanol. Tubes were immediately stored at −80°C until eRNA extraction. Negative filtration control samples (500 mL of distilled water) were collected at the beginning, middle and end of the sampling day, using the same methods, to test whether our contamination prevention procedure during sampling was successful. For each oRNA sample, six live organisms (three adults and three juveniles) per tank were haphazardly collected using a sterile transfer pipette and transferred to a 1.5 mL microcentrifuge tube. We used a P1000 pipette to remove any remaining tank water and then added 370 μL RLT buffer (Qiagen) and 3.7 μL β-mercaptoethanol to the microcentrifuge tube. To maintain the *Daphnia* heat stress transcriptional signal, tubes were flash frozen in liquid nitrogen for 2 s and immediately stored at −80°C until RNA extraction. The following nomenclature was used for sample identification: RNAtype_Temperature&TankID (e.g. sample 'eRNA_20T1' refers to eRNA collected at 20°C from Tank 1).

2.3 | Extractions of eRNA and oRNA samples

Samples of eRNA and oRNA were extracted using the RNeasy Mini Kit (Qiagen), following the manufacturer's protocol with the following modifications. eRNA samples were thawed on ice, gently vortexed for 5 s and then centrifuged for 3 min at 20,000 *g* to separate the liquid from filter. To increase the eRNA yield, liquid from two technical replicates was transferred into a 5 mL microcentrifuge tube containing 800 μL 70% EtOh and pipette mixed. This mixture was transferred to an RNeasy spin column (700 μL at a time) and centrifuged at 8000 *g* for 15 s. We repeated this four times, until all remaining liquid from the 5 mL microcentrifuge tube was transferred to the spin column. For oRNA, *Daphnia* samples were thawed on ice and homogenized via sterile pestle and mortar mixer for 60 s. We then added 400 μL of 70% EtOh to the homogenized tissue. The resulting mixture was pipette mixed and transferred to an RNeasy spin column, where it was centrifuged at 8000 *g* for 15 s. The remainder of the eRNA and oRNA extraction procedures followed the manufacturer's instructions, with RNA being eluted in 40 μL of DNase- and RNase-free molecular grade water.

All eRNA samples were processed in a pre-PCR clean laboratory dedicated to low-quality environmental and ancient DNA/RNA samples. Prior to entering the pre-PCR sterile laboratory, researchers entered a decontamination room to change into the dedicated clean lab coats and put on face masks, hairnets and lab clogs with shoe covers. The laboratory workbench was soaked in a 20% bleach solution for 10 minutes and RNase WiPER™ (iNtRON Biotechnology), and 20% bleach was used to thoroughly wipe pipettes, vortex mixers and centrifuges. Aerosol filter barrier pipette tips were used to prevent cross-contamination of samples. Filtration negative control samples and molecular negative control samples (consisting of reagents or DNA/RNA free molecular grade water) were processed at each step alongside eRNA and oRNA samples and were free of contaminating nucleic acids as identified by failed PCR amplification using universal COI primers known to amplify *Daphnia* DNA (Leray et al., 2013).

2.4 | DNA digestion

Immediately following RNA extraction, an 18 μL aliquot of both RNA types underwent two rounds of DNA digestion using the DNA-*free*™ DNA Removal Kit (Invitrogen), which provided rDNase. We completed the first round of DNA digestion following the manufacturer's instructions, but skipped the DNase inaction step. To ensure no DNA carryover, we completed a second round of DNA digestion as follows: 2.7 μL of DNase I Buffer and 1 μL rDNase were added and pipette-mixed into each sample, and the plate was gently vortexed,

centrifuged and then incubated at 37°C for 20 min. Following incubation, 2 μL of DNase Inactivation Reagent was added to each well. The plate was incubated for 2 min at room temperature and centrifuged at 2000 *g* for 5 min to pellet the DNase Inactivation Reagent. The supernatant containing RNA was transferred to individual sterile microcentrifuge tubes and stored at −80°C until library preparation. Successful DNA digestion of each eRNA and oRNA sample was verified by failed PCR amplification of RNA post-DNA digestion ×2 using universal COI primers known to amplify *Daphnia* DNA (Leray et al., 2013).

2.5 | RNA-seq library preparation and sequencing

We prepared libraries for whole transcriptome shotgun sequencing (RNA-seq) using the Illumina Stranded Total RNA Prep with Ribo-Zero Plus kit (rRNA depletion) and IDT® for Illumina® RNA UD Indexes Set A, following the manufacturer's instructions. Equal volumes of RNA (post 2 rounds of DNA digestion) from each technical replicate were pooled and used as input material (250 ng and 26.4–65.56 ng for oRNA and eRNA, respectively) to prepare one biological replicate library per tank. During library amplification, we used 12 and 17 PCR cycles for oRNA and eRNA, respectively, which followed the instructions outlined by the manufacturer for the respective RNA input amounts. Library quantification, quality control and equimolar pool sequencing of the 16 libraries were conducted at the McGill Genome Centre on one Illumina NovaSeq 6000 S4 lane using paired-end 100 bp reads. RNA-seq libraries were also prepared from negative control samples and were free of contamination as indicated by quality control checks and failed amplification conducted at the McGill Genome Centre.

2.6 | Bioinformatic pipeline and statistical approaches

Raw FASTQ files underwent initial sequencing quality inspection using FastQC (Andrews, 2010). Low-quality sequences and adapters were removed using Trimmomatic (Bolger et al., 2014; ILLUMINACLIP: IlluminaStrandedTotalRNA_adapter.fa:2:30:15 TRAILING:30 HEADCROP:1 MINLEN:90). All statistical analyses were conducted using R (R Core Team, 2021, R version 4.1.2). With default parameters, STAR (Dobin et al., 2013) was used to map all eRNA and oRNA reads that passed quality control and trimming to the *D. pulex* reference genome (Ye et al., 2017). FeatureCounts (Liao et al., 2014) was used on the BAM file output from STAR to quantify gene counts. Only those genes with a sum of ten or more counts in either 20 or 28°C were considered detected and used in the subsequent differential expression analyses, for both eRNA and oRNA. This strict count cut-off potentially lowered the number of genes we could detect and identify as differentially expressed, but we felt was necessary to account for the low eRNA read counts and limit the identification of false-positive differentially expressed genes.

We also examined how the proportion of total reads that mapped to *D. pulex* varied across temperature treatments; this could provide some indication whether eRNA degradation was higher (and thus pseudo-steady-state concentrations potentially lower) in the 28°C treatment. The proportion of *Daphnia* reads relative to the total was modelled as a binomial variable (logit-link) using a generalized linear model, with temperature treatment included as a fixed categorical effect. Significance of the temperature term was evaluated using a χ^2 test, and pairwise comparisons between treatments were evaluated using a *t*-test.

Differential expression analysis was conducted with DESeq2 (Love et al., 2014), comparing all 20°C samples versus all 28°C samples, for both eRNA and oRNA samples. To determine significantly differentially expressed genes (DEG), we used a false discovery rate (FDR) adjusted p-value < 0.05. A Pearson's chi-squared test with Yates' continuity correction was conducted to test for an association between the DEG identified in eRNA and oRNA. WEGO 2.0 (Ye et al., 2018) was used to classify gene ontology (GO) terms (acquired from the reference genome) to all annotated *D. pulex* DEG. GO enrichment analysis was conducted using topGO (Alexa & Rahnenfuhrer, 2022) by comparing GO terms of the relevant genes to the genomic background. A FDR-corrected p-value < 0.05 indicated significant enrichment. A principal component analysis (PCA) of the regularized log (rlog)-normalized eRNA and oRNA gene expression profiles (300 most variant genes) at both optimal and heat-stressed conditions was conducted using DESeq2's plotPCA function (Love et al., 2014). Heatmaps showing the relative expression (Z-score calculated for each gene) of all DEG between 20 and 28°C, in both *Daphnia* oRNA and eRNA, were generated using pheatmap (Kolde, 2019).

We used the SqueezeMeta metatranscriptomics pipeline for the eRNA community-wide analysis (Tamames & Puente-Sánchez, 2019). Briefly, metatranscriptomes were assembled in sequential mode and contigs were assembled using Megahit (Li et al., 2015). Diamond was used to align all contigs to the GenBank nr and KEGG databases for taxonomic assignment and KEGG ID annotation, respectively (Buchfink et al., 2015; Kanehisa & Goto, 2000). We performed differential gene expression analyses as described above for *Daphnia*, after we mapped, using Kallisto (Bray et al., 2016), eRNA reads to the reference genomes and transcriptomes of species known a priori to persist in the communities (*A. falcatus*, *S. quadricauda* and *P. subcapitata*) and highly abundant species identified by SqueezeMeta (*Brachionus plicatilis*, *Volvox carteri*, *Stylonychia lemnae* and *Chlamydomonas eustigma*; Aeschlimann et al., 2014; Han et al., 2019; Hirooka et al., 2017; Nag Dasgupta et al., 2018; Prochnik et al., 2010; Schomaker & Dudycha, 2021; Suzuki et al., 2018). We used the SQMtools R package to further analyse the SqueezeMeta data and created stacked-bar plots to represent the most relatively abundant taxa (Puente-Sánchez et al., 2020). We conducted nonmetric multidimensional scaling analysis of all KEGG IDs to visualize the community-wide functional profiles. We also estimated the similarity of our samples using reads that were unclassified based on the SqueezeMeta analysis. To do this, we randomly extracted five million reads per eRNA sample from unclassified reads. We then used

SimKa to estimate k-mer similarity between eRNA samples (Benoit et al., 2016) and performed multidimensional scaling based on presence–absence using the Bray–Curtis distance matrix.

3 | RESULTS

3.1 | Daphnia pulex gene detection and expression profiles

Across all treatments, 0.51% of eRNA reads and 94% of oRNA reads mapped to the *Daphnia pulex* reference genome (Table S1). However, the mean proportion of mapped eRNA reads differed across temperature treatments ($\chi^2 = 1{,}684{,}536$, $p < 0.001$; Figure S1), with 0.75% and 0.25% of reads in the 20°C and 28°C mapping to *Daphnia*, respectively ($t_6 = 1206$, $p < 0.001$). Sequencing eRNA from tank water and oRNA from *Daphnia* tissue detected 3919 (21%) and 17,244 (93%) genes, respectively, of the 18,449 genes that compose the *D. pulex* reference genome (Ye et al., 2017). All *Daphnia* genes detected in eRNA were also detected in oRNA. A principal component analysis of the regularized log normalized expression for the 300 most variable genes showed separation between experimental groups (Figure 1). The samples were separated across the first principal component (PC1) by RNA type and across the second principal component (PC2) by temperature, clustering eRNA, oRNA, 20°C and 28°C within their respective groups. The only exception was 20°C eRNA sample T1, which unexpectedly clustered closely with the 28°C eRNA group. PC1 and PC2 accounted for 64% of explained variance of all principal components (Figure S2).

3.2 | Daphnia pulex differential gene expression

Comparison of gene expression profiles at 20°C and 28°C identified 32 significantly DEG (false discovery rate-adjusted p-value < 0.05) from eRNA (4 upregulated and 28 downregulated) and 2351 DEG from oRNA samples (1105 upregulated and 1246 downregulated). All eRNA DEG were substantially up and downregulated (exceeding Log_2 2 fold change), as well as 41 and 202 oRNA DEG, respectively. The same directional change (up/downregulated) was observed in 31/32 eRNA DEG as in oRNA (Table S2). Of all eRNA DEG, 17 (2 upregulated and 15 downregulated) were also DEG in oRNA (Figure 2). There was a significant association between DEG identified in oRNA and DEG identified in eRNA ($\chi^2 = 18.26$, df = 1, p-value = 1.92e-05). A heatmap comparing the relative expression (Z-score) of the 17 *Daphnia* DEG common to eRNA and oRNA revealed four distinct clusters based on hierarchical clustering analyses, separating samples and genes by temperature and directional change, respectively (Figure 3). Across both temperature conditions, eRNA and oRNA clustered together and were not separated into RNA types, indicating that eRNA and oRNA exhibit similar levels of relative gene expression for these DEG. The only exception was eRNA 20°C T1 sample which, similar to the PCA, unexpectedly clustered with the 28°C samples.

3.3 | Daphnia pulex functional annotation

Functional enrichment analysis of eRNA detected genes identified 50 GO terms as enriched (FDR-corrected p-value < 0.05), with 26,

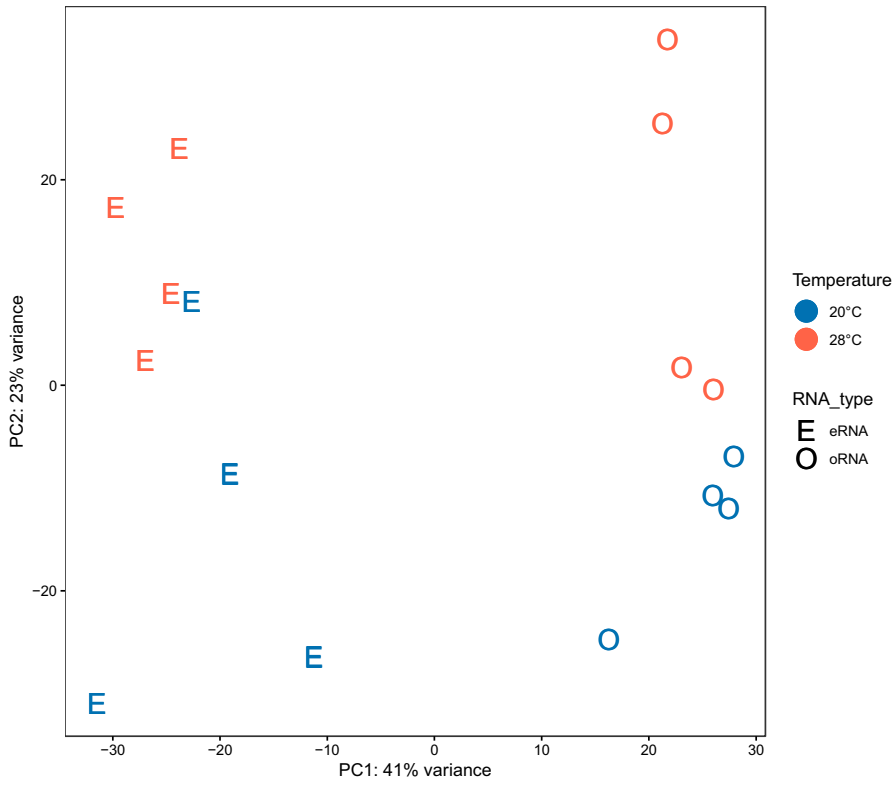

FIGURE 1 Principal component analysis (PCA) of the top 300 *Daphnia pulex* genes, as ranked by variance. eRNA refers to extra-organismal RNA released by *D. pulex* into the environment, whereas oRNA refers to organismal RNA extracted directly from *D. pulex* tissue. Prior to the PCA, genes were filtered to include only those that had a sum ≥10 counts in either 20°C or 28°C. PCA revealed separation by RNA type and temperature across the PC1 and PC2, respectively (except eRNA_20T1 clusters more closely with 28°C samples than with other 20°C samples).

6 and 18 belonging to biological processes (BP), cellular component (CC) and molecular function (MF) domains, respectively (Figure 4 and Figure S3). Of the 32 *Daphnia* DEG identified from eRNA, 21 had GO annotations from the reference genome (Ye et al., 2017; Figures S4, S5). GO enrichment analysis of highly differentially expressed (exceeding Log_2 2 fold change) eRNA genes, and DEG common to both eRNA and oRNA, identified structural constituent of cuticle and chitin metabolic process, as enriched, respectively (FDR-corrected p-value < 0.05; Figure 4).

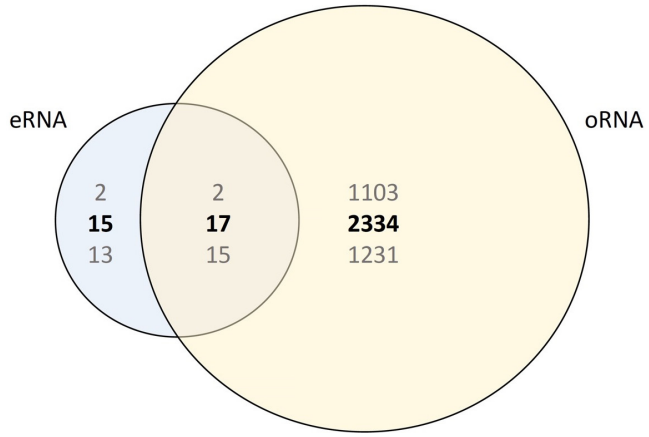

FIGURE 2 Venn diagram showing the significantly differentially expressed *Daphnia pulex* genes (FDR-adjusted p-value < 0.05) between 20°C and 28°C, in both eRNA (blue) and oRNA (yellow) samples. Bold number represents the sum of differentially expressed genes, with up and downregulated genes shown above and below, respectively.

GO enrichment analysis of oRNA detected genes identified 114 GO terms as enriched (FDR-corrected p-value < 0.05), with 100, 1 and 13 belonging to BP, CC and MF domains, respectively (Figure 4 and Figure S3). Of the 2351 DEG identified from oRNA, 1686 had GO annotations from the reference genome (Ye et al., 2017; Figures S4 and S5). GO enrichment analysis of highly differentially expressed (exceeding Log_2 2 fold change) oRNA genes identified 16 GO terms as enriched (FDR-corrected p-value < 0.05), including structural constituent of cuticle, chitin metabolic process, proteolysis, negative regulation of hydrolase activity, negative regulation of proteolysis and response to stimulus (Figure S3).

3.4 | Community composition, differential gene expression and functional annotation

The SqueezeMeta analysis (Tamames & Puente-Sánchez, 2019) identified an average of 43%, 29% and 28% of eRNA reads to be unclassified, microbial coding and noncoding, respectively. Moreover, SqueezeMeta identified 434 taxa, and the community composition and relative abundances remained similar between 20°C and 28°C conditions (Figure S6). Across eRNA samples, an average of 36% of all reads were aligned to eight species, including four eukaryotes that were a priori known to persist in the communities and four highly abundant taxa identified by the SqueezeMeta analysis (Table S1). Of the eight species whose reference genomes we aligned eRNA reads to, we detected 10,026 genes, with the majority of these genes originating from *D. pulex*, *S. quadricauda* and *A. falcatus*, with 3919, 3180 and 1982 genes, respectively (Table S3). Differential gene expression analysis between 20°C

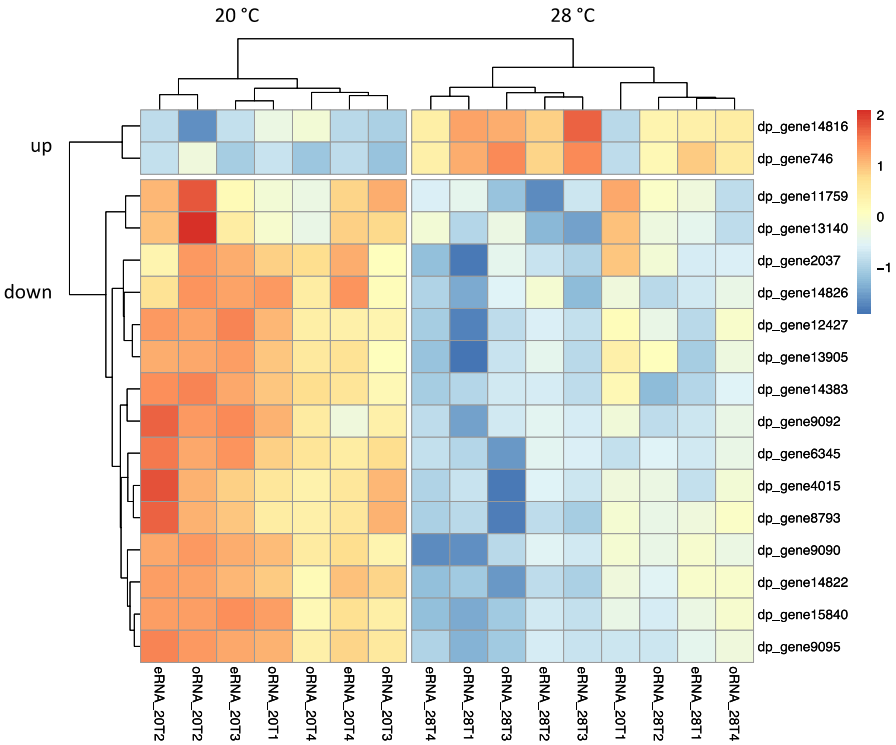

FIGURE 3 Heatmap showing the relative expression (Z-score calculated for each gene) of all commonly significantly differentially expressed (DE) *Daphnia pulex* genes (false discovery rate-adjusted p-value < 0.05) between 20°C and 28°C, in both environmental RNA (eRNA) and organismal RNA (oRNA). DE analysis was conducted only for those genes that had ≥10 counts in either 20°C or 28°C samples. Hierarchical clustering analysis revealed four groups: 20°C and 28°C samples, and up and downregulated genes (except sample eRNA_20T1 clusters with 28°C samples). Colours indicate levels of relative expression, with blue and red indicating low and high, respectively. DE statistics and functional description of the genes are provided in Table S2.

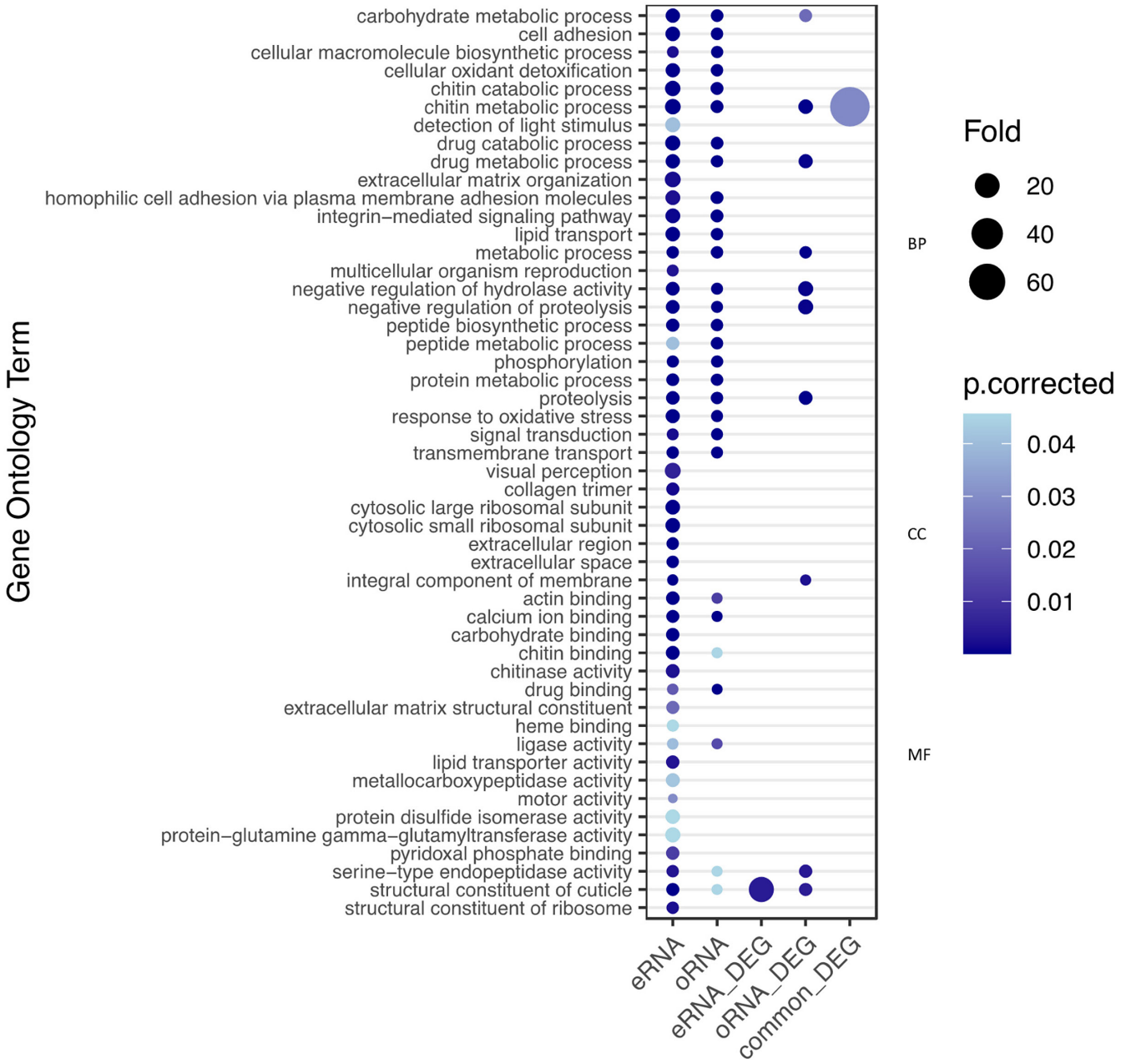

FIGURE 4 Bubble plot of the Gene Ontology (GO) enriched terms associated with *Daphnia pulex* eRNA and oRNA detected genes, and eRNA, oRNA and commonly identified differentially expressed genes (DEG). The eRNA genes originate from extra-organismal *Daphnia* RNA. We used a false discovery rate (FDR) corrected p-value < 0.05 to identify GO terms as significantly enriched compared with the genomic background. The bubble colour indicates the FDR-corrected p-value for the weight test statistic, while the size is proportional to the fold change between expected and observed genes annotated with GO term. The biological processes, cellular components and molecular functions GO domains are represented by BP, CC and MF, respectively. Only those GO terms enriched for eRNA genes are shown. Full list of GO terms enriched for oRNA genes is provided in Figure S3.

and 28°C eRNA samples was performed for each of the eight species and identified a total of 121 significantly DEG (FDR-adjusted p-value < 0.05). The highest numbers of DEG were identified for *A. falcatus*, *D. pulex* and *S. quadricauda* with 44, 32 and 20 DEG, respectively.

Within all eRNA reads, 12,211 KEGG IDs were identified and a nonmetric multidimensional scaling plot of these KEGG IDs revealed distinct functional profiles between 20°C and 28°C communities (Figure S7; stress value = 0.071). Of all KEGG IDs, 93 were identified as significantly differentially expressed (false discovery rate-adjusted p-value < 0.05) between 20°C and 28°C (Table S4). This included KEGG IDs corresponding to proteins and pathways involved in stress responses, such as the p53 signalling pathway, ubiquitin-mediated proteolysis, metabolic pathway and cyclin-dependent kinase 4 (Table S4). Our multidimensional scaling of the Bray–Curtis distance matrix based on unclassified eRNA reads revealed separation

between 20°C and 28°C samples, although one of the latter samples clustered more closely with the former (Figure S8).

4 | DISCUSSION

Conducting transcriptomic analysis of macroorganisms based on extra-organismal eRNA remained previously untested despite recent evidence demonstrating that extra-organismal RNA persists in the environment long enough to be extracted (Kagzi et al., 2022; Littlefair et al., 2022; Marshall et al., 2021; Tsuri et al., 2021; Wood et al., 2020). Our findings revealed that environmental transcriptomics is sensitive in detecting transcriptional heat stress responses even without sampling source macroorganisms directly. From eRNA released by *Daphnia pulex* into the tank water, we detected thousands of *Daphnia* genes and identified a subset of heat stress-relevant genes to be differentially expressed between the two temperature conditions, with levels of relative expression similar to oRNA. We also detected community-wide changes in functional profiles. Our study demonstrates the ability of environmental transcriptomics to reveal gene expression responses of macroorganisms based on extra-organismal eRNA and potentially complex biotic communities, following environmental changes.

4.1 | Recovery of *Daphnia pulex* extra-organismal eRNA

Several studies have recently demonstrated robust detection and persistence of extra-organismal eRNA in the environment (Kagzi et al., 2022; Marshall et al., 2021; Wood et al., 2020). We similarly were able to recover *Daphnia* eRNA in all samples and had an average of 0.51% of all eRNA reads mapping to the *D. pulex* reference genome (Table S1). The best comparison we can currently make of our extra-organismal eRNA read recovery, although not perfect, is to metagenomic studies of natural aquatic ecosystems that found between 0.27% and 1.25% of environmental DNA (eDNA) reads to be potentially of extra-organismal origin (Cowart et al., 2018; Monchamp et al., 2022; Stat et al., 2017; Székely et al., 2021). Our recovery of eRNA was particularly notable given the general expectation that within environmental samples, potentially low abundance eRNA is 'competing' to be sequenced with larger quantities of oRNA from whole microorganisms. This presents a challenge for more complex natural systems with lower population densities of target species than in our experimental *Daphnia* tanks, because macroorganism signals from eRNA could be overwhelmed by an abundance of microorganisms. We preprocessed our libraries with rRNA depletion, but future studies could alternatively conduct poly(A) enrichment to retain only eukaryotic mRNA and eliminate bacterial RNA from eRNA samples. Furthermore, field studies could potentially increase the ratio of macroorganism eRNA and whole microorganisms captured by using large pore filters (i.e. >0.7 μM) and sampling ecosystems densely populated with target macroorganisms (Yates et al., 2021).

In our principal component analysis of *Daphnia* gene expression profiles (Figure 1), eRNA and oRNA samples separate across the first principal component which is unsurprising given the large differences in number of *Daphnia* reads (Table S1). Using the high-quality oRNA samples as a positive control for eRNA, we observe the same separation between temperature conditions except for a single 20°C sample (eRNA_20T1) that clusters more closely to 28°C. Since the precision of transcript quantification increases with sequencing depth (Mortazavi et al., 2008), a possible explanation for this 'wrong' clustering is random technical noise resulting from having too few eRNA reads. This is supported by our heatmap (Figure 3) as the same sample clusters 'wrongly' with 28°C samples, which seems to be caused by most genes having low relative expression levels (i.e. few read counts). However, the upregulated genes and first three rows of downregulated genes exhibit relative expression levels that strongly resemble the 'correct' 20°C conditions. This suggests that the number of *Daphnia* reads for the outlier sample was adequate to quantify some genes, but too low to correctly characterize the overall gene expression profile in Figure 1. We believe that quantification of eRNA gene expression was limited by the number of reads, which could be overcome through deeper sequencing and/or the solutions discussed previously.

4.2 | *Daphnia pulex* extra-organismal eRNA differential gene expression analysis

Using an environmental transcriptomics approach, we were able to detect 3919 *Daphnia* genes from extra-organismal eRNA. As expected, we identified many more (2351) DEG under temperature stress from *Daphnia* oRNA than eRNA (32). This discrepancy in DEG identification can be easily attributed to large differences in the amount of RNA captured that is specific to *Daphnia* among other organisms in the community. This is reflected in the number of reads, 1 and 200 million, that mapped to the *D. pulex* reference genome using eRNA and oRNA, respectively (Table S1). We only included genes in our analysis that had 10 or more read counts in either temperature treatment, which potentially resulted in excluding genes with low counts. However, we felt this cut-off was necessary to limit false-positive detection of genes and false-positive identification of DEG resulting from random noise changes in read counts between temperature conditions.

We were still able to gain ecologically relevant insights from less than 1% of eRNA reads that mapped to *D. pulex*, although this limited our power to detect additional DEG. The low number of *Daphnia* eRNA reads is expected given that the libraries were generated from diverse environmental samples which included RNA transcripts from over 400 taxa, whereas the oRNA libraries were prepared from concentrated *Daphnia* tissue samples. For our proof-of-concept study, we performed rRNA depletion and deep sequencing (average of 158

million reads per eRNA sample, Table S1) in anticipation of such diverse eRNA samples to enable sufficient recovery of *Daphnia* eRNA for gene expression analysis. Despite the lower abundance of *Daphnia* reads from eRNA than oRNA, 97% of eRNA DEG exhibited the same directional change (up/downregulated) as in oRNA (Table S2) and similar levels of relative expression in commonly shared DEG (Figure 3). It is promising that the differential expression response was conserved between oRNA and eRNA despite the latter having far fewer *Daphnia* reads (Table S1), but these results are potentially conditioned by the experimental nature of our study and it is unknown how environmental transcriptomics will translate to natural ecosystems.

A potential issue with eRNA is that it is expected to have far fewer extra-organismal read counts than its oRNA counterpart due to sample origin and influence of external and technical factors on the molecule. The availability of eRNA is likely influenced by factors such as abiotic and biotic conditions, rate and mechanism of release and transport (Cristescu, 2019). Technical factors such as capture and extraction methods that are known to impact eDNA recovery (Deiner et al., 2015) are also likely to influence eRNA recovery. Future studies investigating the ecology of eRNA and how technical factors influence eRNA recovery and read counts are necessary to clarify how researchers may interpret eRNA results from complex natural ecosystems (See Cristescu, 2019; Yates et al., 2021 for outstanding questions).

It is important to consider the temperature treatments when interpreting our differential expression results because eRNA might degrade more rapidly in warm waters (Jo et al., 2022), which could impact the detection of both up and downregulated genes in temperature experiments. Indeed, we recovered fewer *Daphnia* reads at 28°C than 20°C (Table S1 and Figure S1), suggesting that the pseudo-steady-state concentration of extra-organismal eRNA is lower relative to microbial reads at higher temperatures, either as a result of faster eRNA degradation, higher microorganism abundance or both. However, we accounted for differences between samples in the number of reads that mapped to *D.pulex* using DESeq2's median of ratios method, which normalizes for sequencing depth (Love et al., 2014). Nevertheless, the identification of upregulated genes could be muted in eRNA due to increased degradation at higher temperatures. This potentially explains the discrepancy in the proportion of up and downregulated genes identified in eRNA (4 and 28) and oRNA (1105 and 1246) indicating that several lowly upregulated genes were not identified in eRNA, though DEG identification was also likely influenced by having fewer *Daphnia* reads in eRNA than oRNA. It is possible that downregulated genes with low read counts are not detectable in eRNA; we used a strict 10 count cut-off to limit false-positive gene detections, which could have potentially caused nondetections for genes with read counts below the cut-off. Downregulated genes with low counts could be false positives due to increased eRNA degradation at higher temperatures, but we accounted for this potential bias by normalizing for differences in *Daphnia* read counts. We found a significant association between the DEG identified in eRNA and in oRNA, and that all downregulated eRNA genes were also downregulated in oRNA (Table S2). Therefore, although we potentially missed lowly up and downregulated genes due to eRNA degradation, our results suggest that eRNA mirrored oRNA signals and that the genes we identified as differentially expressed were true positives and not caused by differing eRNA degradation rates.

Changes in gene expression are often observed before phenotypic and demographic effects occur and may therefore act as an early indication of ecological stress (Fedorenkova et al., 2010; Jovic et al., 2017; Snell et al., 2003). We found that eRNA and oRNA recovered similar *Daphnia* chronic heat stress responses, which consisted of downregulation of genes associated with metabolic and cellular processes, membrane, binding, extracellular region, collagen and cuticle structure (Figure 4 and Figure S3). These results are consistent with studies that also found chronically heat-stressed *Daphnia* to exhibit widespread downregulation of genes with similar functions (Becker et al., 2018; Yampolsky et al., 2014). This widespread downregulation of metabolic genes has been hypothesized to be a molecular compensatory mechanism for *Daphnia* at near-lethal temperatures to sacrifice their long-term fitness for immediate survival (Yampolsky et al., 2014).

After 7 days of heat exposure, only few (4/62) heat shock genes were slightly upregulated ($\log_2$ fold change = 1.1–1.3; Table S5) in *Daphnia* oRNA. This aligns with findings suggesting heat shock genes to be less important for the chronic (days) *Daphnia* heat stress response, but central for mitigating cell damage following acute (hours) heat exposure (Becker et al., 2018; Yampolsky et al., 2014). Multiple tropical marine fish species similarly do not differentially express heat shock genes during chronic heat stress (Veilleux et al., 2015), suggesting that heat shock genes might not be optimal for assessments of communities under chronic heat stress. Furthermore, we detected chitin-related GO terms to be commonly enriched in both eRNA and oRNA DEG (Figure 4), corroborating *Daphnia* studies that linked chitin genes to environmental stressors (Becker et al., 2018; Chain et al., 2019; Connon et al., 2008; Shaw et al., 2007). Hydrolytic- and proteolytic-related GO terms were enriched in eRNA (Figure 4) and are commonly involved in *Daphnia* and copepod heat stress response by stabilizing and cleaning up misfolded proteins (Becker et al., 2018; Kelly et al., 2017). It is promising that using eRNA, we were able to detect the widespread downregulation of genes associated with chronic heat stress with similar levels of relative expression as oRNA. We also found that many GO terms were commonly shared in both RNA types and that stress-related terms were enriched in eRNA. Collectively, our results indicate that environmental transcriptomics based on extra-organismal eRNA is able to detect heat stress responses of source macroorganisms through changes in gene expression and functional GO terms. Future empirical work is necessary to determine how our results extend to more complex natural ecosystems.

4.3 | Community composition and function

We found the community composition to remain largely unchanged for 20°C and 28°C conditions, after mapping all eRNA reads to

GenBank nr (Figure S6). Since we prefiltered the tank water at 60 μm to remove *Daphnia*, all non-*Daphnia* RNA recovered in our eRNA samples likely originates from whole microorganisms that passed the filter. Within our eRNA samples, gene expression analysis of eight species detected 10,026 genes with 121 of these being significantly differentially expressed between 20°C and 28°C (Table S3). Although community compositions remained similar, analysis of KEGG orthologs revealed distinct functional profiles for communities in the two temperature treatments (Figure S7). We found stress-related KEGG orthologs to be differentially expressed, including proteins involved in binding, kinase and oxidoreductase (Table S4). Our community-wide results reflect similar shifts in functional profiles observed in conventional aquatic metatranscriptomic studies based on bulk microorganism samples (Aylward et al., 2015; Frias-Lopez et al., 2008; Gilbert et al., 2008; Marchetti et al., 2012; Moniruzzaman et al., 2017; Poretsky et al., 2005; Salazar et al., 2019; Vorobev et al., 2020). For example, a freshwater metatranscriptome likely under heat stress exhibited similar stress responses, which included oxidoreductase, binding and kinase proteins (Trench-Fiol & Fink, 2020). We build upon this rich metatranscriptomics literature by demonstrating that in addition to yielding microorganism insights, environmental transcriptomics can also noninvasively detect the gene expression response of macroorganisms to environmental stress.

4.4 | Recommendations and future directions

Interest in nonlethal transcriptomics is growing, as such an approach increases animal welfare, allows for repeated survey of individuals and linking gene expression to fitness following exposure to stress (Czypionka et al., 2015; Jeffries et al., 2021; Veldhoen et al., 2014). However, conventional nonlethal methods are species-specific and involve animal handling and sampling for blood or tissue, which may still result in organism stress and reduced survival upon release into the environment (Martins et al., 2018; Portz et al., 2006; Young et al., 2019). Inferring the physiological state of organisms from noninvasive eRNA samples would circumvent these stressful sampling procedures and represent a substantial improvement for animal welfare. We demonstrated that similar to the conventional oRNA approach, environmental transcriptomics based on extra-organismal eRNA can noninvasively detect heat stress responses of progenitor macroorganisms.

An exciting prospect of environmental transcriptomics is the potential to monitor the health of complex communities, including multiple macroorganisms. Biomonitoring traditionally relies upon identifying bioindicator taxa that are correlated with environmental conditions, but transcriptomic surveys can provide valuable early warnings since gene expression changes are likely to occur before compositional shifts could be detected (Veilleux et al., 2021). Conventional metatranscriptomics has demonstrated that gene expression profiling of microorganisms reflects environmental conditions (Marchetti et al., 2012; Moran et al., 2013; Salazar et al., 2019; Shi et al., 2011). Our results suggest that environmental transcriptomics could extend gene expression analyses beyond microorganisms and has the potential to provide functional information of species across the trophic chain as eRNA includes extra-organismal RNA from a diversity of macroorganisms. However, we currently do not have a full understanding regarding the feasibility of environmental transcriptomic analysis of multiple macroorganisms in complex natural ecosystems.

Empirical tests of environmental transcriptomics targeting macroorganisms in more complex systems and in nature are needed before being applied for biomonitoring. Controlled mesocosms populated with multiple macroeukaryotes (whose genomes are sequenced) and at varying densities are logical next-step experiments and would enable researchers to align eRNA reads to available reference genomes and to conduct differential gene expression analyses as we did with *Daphnia*. Moving to the field and using existing metatranscriptomic pipelines with eRNA could be challenging if macroeukaryote presence is not a priori known because many reads may remain unclassified and/or genes may not be assigned to a single species due to limited reference databases. These unclassified reads could potentially provide insights into community responses to environmental change as demonstrated by our unclassified reads clustering primarily by temperature treatments (Figure S8). In situations where reads cannot be assigned to a single species, it may only be possible to conduct analyses at higher taxonomic orders and not at the species level. Development of genomic resources that enable accurate mapping of reads and definition of gene expression for many macroeukaryotes of interest would therefore be useful.

Based on what we observe in our experiment, environmental transcriptomics is likely to be of limited use for low-density/rare species in an ecosystem. This approach is potentially best suited for circumstances where density is high, that is for organisms that form breeding aggregates (e.g. salmon) or organisms inhabiting habitat patches at high density (e.g. anurans in small ponds scattered throughout the landscape). Systems densely populated with macroeukaryotes whose genomes are sequenced represent an attractive scenario to run field tests of environmental transcriptomics for macroorganisms. Population-level assessments that couple eRNA with targeted approaches (e.g. qPCR and ddPCR) and stress biomarkers are more realistically applicable in the short term.

We anticipate multiple challenges for environmental transcriptomic biomonitoring in natural environments, such as achieving adequate sequencing depth of eRNA in a cost-efficient manner. In complex communities, it is possible that macroeukaryotic mRNA could represent an even smaller fraction of all eRNA than we observed with *Daphnia* (Table S1) and could be overwhelmed by RNA from microorganisms. Thus, conventional metatranscriptomics studies that target microorganisms may not recover enough macroorganismal eRNA for analysis. Environmental transcriptomic surveys should therefore be designed with the intention of targeting macroeukaryotes and could conduct poly(A) enrichment to retain only eukaryotic mRNA in libraries (Zhao et al., 2018).

Sequencing technologies are also rapidly advancing and the costs continue to decrease (Wetterstrand, 2021) which will enable future studies to generate more reads less expensively, thereby increasing abundance of extra-organismal eRNA transcripts. Additionally, the continued development of low-input mRNA-seq library preparation kits will enable researchers to prepare libraries for environmental transcriptomics with minimal template eRNA. Molecular technological advancements that enhance extraction efficiency and remove ribosomal RNA from diverse samples may increase the yield of coding eRNA and thus the power of environmental transcriptomics.

4.5 | Conclusions

By providing functional insights in a noninvasive manner, eRNA-based approaches could push beyond the limitations of eDNA species detection and conventional tissue-based transcriptomic surveys. We demonstrated that environmental transcriptomics based on extra-organismal eRNA from macroorganisms can detect thousands of genes and that a subset of functionally relevant genes can be identified as differentially expressed under heat stress, with levels of relative expression similar to the conventional oRNA approach. Within all eRNA reads, we were able to reveal changes in functional profiles for the community in response to heat stress. Collectively, these findings demonstrate the ability of eRNA to detect changes in gene expression of progenitor macroorganisms following environmental stress and the potential of environmental transcriptomics to provide functional information across the trophic chain via extra-organismal eRNA. Future empirical work is necessary to determine if this approach can be extended to natural ecosystems.

AUTHOR CONTRIBUTIONS

RMH and MEC designed the study. RMH and MCY conducted the experiment. RMH conducted all molecular work. RMH and FJJC performed the bioinformatic analysis and produced the figures. RMH wrote the first draft of the manuscript and all authors contributed to editing the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

OPEN RESEARCH BADGES

This article has earned an Open Data Badge for making publicly available the data necessary to reproduce the reported results. Sequences for all RNA-seq libraries have been uploaded to the NCBI SRA under BioProject PRJNA830892. Scripts are available at https://doi.org/10.5281/zenodo.8218040.

DATA AVAILABILITY STATEMENT

Sequences for all RNA-seq libraries have been uploaded to the NCBI SRA under BioProject PRJNA830892. Scripts are available at https://doi.org/10.5281/zenodo.8218040.

ORCID

Robert M. Hechler https://orcid.org/0000-0002-9961-7179
Matthew C. Yates https://orcid.org/0000-0002-9199-1078
Frédéric J. J. Chain https://orcid.org/0000-0001-6169-7399

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