Different ways to play it cool: Transcriptomic analysis sheds light on different activity patterns of three amphipod species under long-term cold exposure

Polina Lipaeva1 | Kseniya Vereshchagina2,3 | Polina Drozdova2,3 | Lena Jakob4 | Elizaveta Kondrateva2 | Magnus Lucassen4 | Daria Bedulina2,3 | Maxim Timofeyev2,3 | Peter Stadler5,6,7,8,9 | Till Luckenbach1

1Department of Bioanalytical Ecotoxicology, Helmholtz Centre for Environmental Research—UFZ, Leipzig, Germany
2Institute of Biology, Irkutsk State University, Irkutsk, Russia
3Baikal Research Centre, Irkutsk, Russia
4Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Bremerhaven, Germany
5Bioinformatics Group, Department of Computer Science, Interdisciplinary Center for Bioinformatics, Universität Leipzig, Leipzig, Germany
6Max Planck Institute for Mathematics in the Sciences, Leipzig, Germany
7Department of Theoretical Chemistry, University of Vienna, Vienna, Austria
8Facultad de Ciencias, Universidad Nacional de Colombia, Bogotá, Colombia
9Santa Fe Institute, Santa Fe, New Mexico, USA

Correspondence
Polina Lipaeva, Department of Bioanalytical Ecotoxicology, Helmholtz Centre for Environmental Research—UFZ, Leipzig, Germany.
Email: polina.lipaeva@ufz.de

Abstract
Species of littoral freshwater environments in regions with continental climate experience pronounced seasonal temperature changes. Coping with long cold winters and hot summers requires specific physiological and behavioural adaptations. Endemic amphipods of Lake Baikal, *Eulimnogammarus verrucosus* and *Eulimnogammarus cyaneus*, show high metabolic activity throughout the year; *E. verrucosus* even reproduces in winter. In contrast, the widespread Holarctic amphipod *Gammarus lacustris* overwinters in torpor. This study investigated the transcriptomic hallmarks of *E. verrucosus*, *E. cyaneus* and *G. lacustris* exposed to low water temperatures. Amphipods were exposed to 1.5°C and 12°C (corresponding to the mean winter and summer water temperatures, respectively, in the Baikal littoral) for one month. At 1.5°C, *G. lacustris* showed upregulation of ribosome biogenesis and mRNA processing genes, as well as downregulation of genes related to growth, reproduction and locomotor activity, indicating enhanced energy allocation to somatic maintenance. Our results suggest that the mitogen-activated protein kinase (MAPK) signalling pathway is involved in the preparation for hibernation; downregulation of the actin cytoskeleton pathway genes could relate to the observed low locomotor activity of *G. lacustris* at 1.5°C. The differences between the transcriptomes of *E. verrucosus* and *E. cyaneus* from the 1.5°C and 12°C exposures were considerably smaller than for *G. lacustris*. In *E. verrucosus*,
cold-exposure triggered reproductive activity was indicated by upregulation of respective genes, whereas in E. cyaneus, genes related to mitochondria functioning were upregulated, indicating cold compensation in this species. Our data elucidate the molecular characteristics behind the different adaptations of amphipod species from the Lake Baikal area to winter conditions.

KEYWORDS
cold adaptation, Eulimnogammarus cyaneus, Eulimnogammarus verrucosus, freshwater lake, Gammarus lacustris, Lake Baikal, transcriptome sequencing

1 | INTRODUCTION

Water temperature is a pervasive abiotic factor for aquatic ectotherms whose body temperature is determined by the ambient temperature. The thermal window’s lower boundary is expanded in cold-adapted species, allowing them to thrive in low-temperature environments. Cold adaptation involves several cellular processes and adjustments, enabling long-term survival at low temperatures.

Depending on the overwintering strategy, ectotherms show various metabolic activity states at low temperatures, ranging from metabolic depression to high activity, including even reproduction (Elgmork, 1991; Graham & Hop, 1995; Schaefer, 1977). The metabolic activity state of aquatic ectotherms at cold temperatures also depends on other parameters, such as availability of food and water oxygen levels (Somero et al., 2017). To lower the energy demand in the cold season, ectotherms often decrease their physiological activity; metabolic rates can be depressed to various degrees, which may lead to semi-torpid or torpid stages (Jackson & Ultsch, 2010; Radzikowski, 2013; Ultsch, 1989; Walsh et al., 1983). In aquatic systems, apart from low temperature, reduction in metabolic activity can be due to shortage in food supply, as well as hypoxia or anoxia as a result of ice cover and decreased photosynthetic activity of water plants. Some ectotherms remain metabolically active at low temperatures during the winter by compensating the biological effects of the cold. This is facilitated by mechanisms of cold adaptation: membrane fluidity adjustments (Bowler, 2018); an increase in mitochondrial density and enzyme capacities (Fangue et al., 2009; Lucassen et al., 2006; Pörtner, 2002); synthesis of cold-adapted enzymes (Fields & Somero, 1998; Genicot et al., 1996); expression of ice-binding proteins (Duman, 2015; Kristiansen & Zachariassen, 2005); and synthesis of polyol cryoprotectants (Joanisse & Storey, 1994; Storey et al., 1991). Most ectotherms in regions with cold winters reproduce in spring. This strategy is widely thought to be selected for so that the initial life phases of the free-living offspring take place under maximally favorable conditions with regard to temperature and food availability (Ultsch, 1989). Reproduction involves high energy consumption. Thus, reproduction during the winter is exceptional among ectotherms from regions with high seasonal temperature changes (Elgmork, 1991; Schaefer, 1977).

Lake Baikal is a UNESCO world heritage site inhabited by one of the richest freshwater faunas in the world. More than 80% of the animal species are endemic (Timoshkin et al., 2001) and adapted to the specific conditions of the lake, such as low mineralization and high oxygen content of the water and ice cover for several months per year (Cabello-Yeves et al., 2018; Kenny et al., 2019; Sideleva, 1996; Zerbst-Boroffka, 1999). The water temperature in the littoral zone of Lake Baikal varies significantly depending on the location, water depth, and time of the year. In the winter-spring period, when the water is covered by ice, the temperature is close to 0°C; in summer and autumn, it ranges from 5°C to up to 18°C (Figure 1), with occasional substantial temperature shifts within hours or a few days due to storm and upwelling events.

Lake Baikal hosts over 350 endemic amphipod species and subspecies that make up approximately 90% of its benthic biomass (Takhteev, 2019). Eulimnogammarus verrucosus (Gerstfeldt, 1858) and Eulimnogammarus cyaneus (Dybowsky, 1874) studied here are endemic amphipod species of Lake Baikal’s littoral zone. Gammarus lacustris Sars, 1863, a widespread representative of the Holarctic fauna, is found in ponds in close vicinity to Lake Baikal and in isolated bays of the lake but almost never at sites inhabited by the endemic Baikal fauna (Takhteev, 2019).

The amphipods studied here differ with regard to their physiological upper thermal limits (Axenov-Gribanov et al., 2016; Jakob et al., 2016) and the temperatures of the water at which they preferentially linger (Timofeyev et al., 2001; Table 1). Eulimnogammarus verrucosus is a cold-loving species found in water depths of 0–15 m; it migrates to deeper waters when water temperatures rise in the upper littoral in summer (Jakob et al., 2016; Weinberg & Kamaltynov, 1998). Eulimnogammarus cyaneus is more thermostolerant than E. verrucosus and inhabits the upper littoral throughout the year. Field observations revealed that E. verrucosus and E. cyaneus maintain high locomotor activity at low temperatures (Vereschchagina et al., 2021) and E. verrucosus was found to reproduce during winter (Gavrilov, 1949). Gammarus lacustris has a slightly higher upper thermal limit than the Baikal endemic E. cyaneus. It is a cold-resistant ectotherm of inland water ecosystems of the Holarctic that prepares for the cold season by the formation of cryoprotective biomolecules (Karanova & Andreev, 2010) and shows hypometabolism and consequently
drastically reduced locomotor activity upon long-term cold exposure (Vereshchagina et al., 2021). *Gammarus lacustris* can be found inactive in leaf litter in winter and spring (own field observations). The molecular bases of the major activity changes in *G. lacustris* in hibernation mode have so far not been explored.

Reference-free transcriptome assemblies from next-generation sequencing data enable acquisition of information about the transcriptome activity of non-model organisms (Cahais et al., 2012). In this study, we analysed transcriptomes of the two Baikal amphipods *Eulimnogammarus verrucosus* and *E. cyaneus* and the Holarctic *G. lacustris* exposed to 12°C, the mean water temperature of the Baikal littoral at 1 m depth in summer, and 1.5°C, the mean temperature in winter (Figure 1). We hypothesized that the transcriptomic profiles in each species will mirror the species-specific metabolic activity patterns in those temperature scenarios. Here, we aimed to identify hallmarks of (1) metabolic depression observed at this temperature in *G. lacustris*; (2) the high metabolic activity of the *Eulimnogammarus* species kept at this temperature; and (3) the onset of reproductive processes in *E. verrucosus* in the transcriptomic responses in the animals upon exposure to low temperature (1.5°C).

**2 | MATERIALS AND METHODS**

**2.1 | Sampling and experimental design**

*Eulimnogammarus verrucosus* (Gerstfeldt, 1858) and *Eulimnogammarus cyaneus* (Dybowsky, 1874), endemic to Lake Baikal, were caught with a hand-net by kick-sampling in the littoral zone at water depths of 0.5–0.8 m close to the Biological Station of Irkutsk State University in Bolshie Koty (south-west Baikal). *Gammarus lacustris* Sars, 1863 was sampled in a former gold mining pond ("Lake №14": 51°55′14.39″N, 105°4′19.48″E) in about 2 km distance from the Biological Station and Lake Baikal. There are no obvious signs of toxic contamination of this pond, for example with heavy metals, as it is inhabited by various biota, such as *G. lacustris* and aquatic insects (Odonata, Diptera, Coleoptera, and Hemiptera) that are highly abundant. Fresh water constantly seeps into the pond from the underground. The temperature of the water at the bottom of the pond (1–1.5 m water depth) is rather stable at 7–8°C during the summer (own observations).

**FIGURE 1** Water temperature regimes in the habitat of the amphipods *E. verrucosus* and *E. cyaneus* from Lake Baikal. The black dots represent the mean daily water temperatures in the Baikal littoral zone at 1 m water depth from July 2016 until the beginning of June 2017. For better visualization, the dots were connected with a black line. Temperatures were monitored every 0.5 to 3 h with a data logger (no. DS1922L, iButton, Maxim Integrated, CA, U.S.) mounted to a wooden pillar at the lake shore at Bolshie Koty (south-west Baikal). Dashed red and blue lines indicate the experimental exposure temperatures in this study (see below).

**TABLE 1** Temperatures (preferred, LT50, TpII) indicating the physiological temperature ranges of the Baikal amphipods *E. verrucosus* and *E. cyaneus* and the Holarctic *G. lacustris*; TpII, pejus temperature ("pejus" = Latin for "getting worse")

| Species          | Preferred T, °C | LT50, °C | TpII, °C | Activity at a temperature close to zero |
|------------------|-----------------|----------|----------|----------------------------------------|
| *E. verrucosus*  | 5–6°            | 21.1–21.5°, 29–29.5° | 10.6°   | Active, reproducing                       |
| *E. cyaneus*     | 11–12°          | 28.4–30.4° | 19.1°   | Active                                  |
| *G. lacustris*   | 15–16°          | 28.3–30.3° | 21.1°   | Torpor                                  |

*Long-term experiment, 4 week, 0.8°C/day (Jakob et al., 2016).  
*Short-term experiment, 24 h, 1°C/h (Axenov-Gribanov et al., 2016).  
*Timofeyev et al., 2001.  
*Timofeyev & Shatilina, 2007.  
*Vereshchagina et al., 2021.
Animals were caught in August 2016. No specific permissions were required for collecting the studied species at the sampling sites. The species are highly abundant and not endangered or protected.

After sampling, the animals were placed in a cold box with water from the sampling site and transported to the laboratory. The species were separated and rinsed with water from the respective sampling site. In the laboratory, animals were placed in 2 L glass tanks filled with Baikal water (E. verrucosus and E. cyaneus) or with filtered water from Lake №14 (G. lacustris). During acclimation to laboratory conditions and throughout the experiment, the animals were kept in water from these sources. Six tanks with E. cyaneus with 200 individuals each, four tanks with E. verrucosus with 30 individuals each and six tanks with G. lacustris with 75 individuals each were set up. The different numbers of individuals for each species were due to the different body sizes of each species (see e.g., Jakob et al., 2016). The water in the tanks was exchanged at least once every three days. During the experiment, Baikal amphipods were fed ad libitum with a mix of ground dried amphipods, algae, aquatic plants, and detritus collected from the Baikal littoral. Individuals of G. lacustris were fed ad libitum with leaves and detritus collected from Lake №14 and a commercial food mix consisting of dried Gammarus sp. (Barrom, Barnaul, Russia).

Prior to the exposures to 1.5°C and 12°C, the animals were acclimated to laboratory conditions at 6°C, the mean annual water temperature in the Baikal littoral (Falkner et al., 1991; Weiss et al., 1991; Yoshioka et al., 2002) for two weeks. The water temperature in the tanks with the animals was gradually adjusted to 6°C at a rate of 1–1.5°C/day from the water temperature at the respective sampling site (7°C for E. verrucosus and E. cyaneus; 13°C for G. lacustris). After keeping the animals at 6°C for two weeks, the water temperature in half of the tanks (i.e., two tanks for E. verrucosus, three tanks for E. cyaneus and G. lacustris) was gradually decreased to 1.5°C at a rate of 1°C/day over about 6 days. In the other half of the tanks, the water temperature was gradually increased to 12°C by 1.5°C/day over about six days. The amphipods were kept at 1.5°C or 12°C for one month (Figure 2). Upon the one month exposure, the animals were frozen in liquid nitrogen. The tissue of the frozen animals was ground in a mortar to a coarse granule that was stored at ~80°C until further sample processing. Prior to tissue grinding animals were pooled; pools of E. verrucosus, E. cyaneus, and G. lacustris contained tissue of three, 12–15, and 10–12 animals, respectively. Different pool sizes were due to different species-specific body sizes of the studied animals.

Muscle tissue was obtained from E. verrucosus individuals acclimated to 12°C for four weeks. Muscle tissue was dissected in a Petri dish on ice by first removing the head and the urosome from the animals and then cutting open the exoskeleton at both sides along the anterior/posterior axis. The muscle tissue was then detached from the exoskeleton with forceps, placed in a cryotube, and immediately frozen in liquid nitrogen.

2.2 | RNA extraction, library preparation, and transcriptome sequencing

The coarsely ground amphipod tissue was homogenized in 1 ml Qiazo1 reagent (Qiagen) using a MM400 mixer mill (Retsch). Total RNA of amphipods was isolated using the RNeasy Mini kit (Qiagen) and treated with DNase (TURBO DNA-Free Kit, Thermo Fisher Scientific) according to the manufacturer's instructions. mRNA was separated from total RNA using the Oligotex mRNA Mini Kit (Qiagen). RNA concentration and quality were evaluated using a Qubit 2.0 fluorometer (Thermo Fisher Scientific) and a 2100 Bioanalyzer instrument (Agilent).

A total of 24 RNA-seq libraries (three species, two treatments, four replicates) were prepared, each from 20 ng of mRNA using the ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicentre, Madison, WI, USA). In addition, two RNA-seq libraries were prepared from 1 µg of total RNA from muscle tissue of each of two E. verrucosus individuals using the NEBNext Ultra II Directional Library Preparation Kit following the standard protocol for mRNA enrichment with Poly-A selection module (New England Biolabs Ltd.). Paired-end (PE) sequencing was performed with an Illumina HiSeq 2000 device (llumina Inc.), yielding sequences with a read length of 100 bp. The 24 libraries were pooled and sequenced on four lanes of a v3 PE HiSeq flow cell. Two RNA-seq libraries from E. verrucosus muscle tissue were pooled with 22 RNA-seq libraries (from amphipods from a different experiment not analysed in this study) and sequenced on four lanes of a v3 PE HiSeq flow cell.

2.3 | Read quality control and de novo transcriptome assembly

Raw Illumina reads were examined using the quality control tool FastQC (Andrews, 2017) version 0.11.4, and the results were summarized with MultiQC (Ewels et al., 2016) v1.8. Adapters were removed using Trim Galore! version 0.6.5 (Krueger, 2019). The FastQC analysis revealed that up to 16% and 5% of the overrepresented sequences were ribosomal RNA (rRNA) in the whole body (ScriptSeq) and muscle (NEBNext) libraries, respectively. rRNA was removed from the data set with read aligner bowtie2 (Langmead & Salzberg, 2012) version 2.2.6 using a custom database of gammarid rRNA sequences manually extracted from the National Centre for Biotechnology Information (NCBI) Nucleotide database. Rcorrector (Song & Florea, 2015) was then applied to fix random sequencing errors in Illumina RNA-seq reads. Subsequently, de novo transcriptome assembly was performed using Trinity (Grabherr et al., 2013) version 2.9.1 with the --SS_lib_type FR parameter, indicating the strand specificity of the libraries (the first read represents the cDNA strand corresponding to the original RNA strand; the second read corresponds to the respective reverse complement sequence strand of the cDNA). Sequence data from all eight whole body transcriptome...
2.4 | Assembly quality control, filtering, and annotation

Assembly quality was assessed with TransRate (Smith-Unna et al., 2016) version 1.0.3 and assembly completeness with the Benchmarking Universal Single-Copy Orthologues (BUSCO) tool (Simão et al., 2015) version 4.0.4 using the arthropoda_odb10 data set of 1066 single-copy orthologues of arthropods. The proportion of reads mapped to the assembly was estimated with bowtie2 (Figure S1).

To avoid redundant transcripts, assembled contigs were clustered with cd-hit-est (Li & Godzik, 2006) version 4.8.1 with 95% sequence identity threshold and parameter -n 10 (word length). Misassembled or incomplete contigs were filtered out from nonredundant assemblies using TransRate and the assembly quality was then assessed using bowtie2 and the BUSCO tool (Figures S1 and S2A).

Homology searches of filtered assemblies were performed using DIAMOND (Buchfink et al., 2015) version 0.9.24.125 with its sensitive mode against the NCBI nonredundant protein sequence database of 2 March 2020. DIAMOND provides taxonomic assignment of the annotated contigs and only the contigs assigned to metazoan species were selected.

Downstream analyses were performed on the filtered contigs. Functional annotation was done with the eggNOG-mapper v2 web server (Huerta-Cepas et al., 2017), using the eggNOG v5 database (Huerta-Cepas et al., 2019). Orthologous assignment and mapping of the unigenes to the biological pathways were conducted with the KAAS-KEGG web server with default BLAST settings (Moriya et al., 2007).

2.5 | Transcript abundance and differential expression analysis

Transcript abundances in the transcriptomes were analysed with the align_and_estimate_abundance.pl script from the Trinity toolkit. Alignment-free quantification was conducted with the abundance estimation tool salmon (Patro et al., 2017) version 1.1.0. We used the R package tximport (Soneson et al., 2016) version 1.14.2 to import transcript lengths and abundance estimates into matrices for the following statistical analysis. Differential expression was estimated based on the count matrix generated with DESeq2 (Love et al., 2014) version 1.26.0, which pre-processes gene expression data and runs the Wald test on log2 fold change (LFC) values in order to statistically assess differences between treatments based on all replicates. The Benjamini-Hochberg method was used to select a set of significantly differentially expressed (DE) genes with a false discovery rate, that is, adjusted p-value threshold, of .05. Gene set enrichment analysis of functionally annotated DE genes was conducted using fgsea (Korotkevich et al., 2019) version 1.12.0 for R. For pathway-based data integration and visualization, the R package pathview (Luo & Brouwer, 2013) version 1.26.0 was used.

3 | RESULTS

3.1 | Mortalities and behavioural observations of the amphipods in the temperature treatments

Mortalities were 0.3% in the 12°C and zero in the 1.5°C treatment for E. verrucosus and 1.9% at 1.5°C and 0.8% at 12°C for G. lacustris. No mortality was detected for E. cyaneus in either treatment.

Throughout the exposure period, E. cyaneus and E. verrucosus individuals were found to be actively moving in both temperature treatments. In contrast, G. lacustris individuals were only actively
moving in the 12°C treatment but showed little locomotor and feeding activity at 1.5°C, mostly lying on the tank bottoms.

3.2 | De novo transcriptome assemblies

Reference transcriptomes from de novo assembled sequence reads comprised 712,077; 833,922; and 603,270 contigs for G. lacustris, E. verrucosus, and E. cyaneus, respectively (refer to Table S1 for technical characteristics). To avoid redundant transcripts and mis-assembled contigs, which potentially confound data interpretation, duplicated transcripts with sequence similarities >95% and assembly artefacts were excluded; upon filtering of data, between 61.5% and 70.7% of assembled transcripts were retained (Table 2).

BUSCO results revealed that more than 90% of the 1066 near-universal Arthropoda orthologues were present in the nonfiltered transcriptome assemblies (Figure S2A). However, only about 30% of the orthologues were identified as complete single copies. The filtering of the assemblies led to an almost two-fold increase in complete single-copy BUSCO groups. The rest of the duplicated genes (~10%–30%) are likely to result from multiple haplotypes, alleles or isoforms.

3.3 | Annotation and differential gene expression

The contigs obtained here were annotated by homology using the NCBI nonredundant protein sequence database. About 30% of contigs (138,990; 187,714; and 99,627 contigs for G. lacustris, E. verrucosus, and E. cyaneus, respectively) showed significant similarities to known proteins; about 70% of contigs could not be annotated. From the annotated contigs, 29% (E. verrucosus), 44% (E. cyaneus), and 44% (G. lacustris) were assigned to metazoan species; 44%–52% of the contigs were from bacteria and protozoa (Figure S2B).

Amphipods are known to host numerous symbionts, commensals, and pathogens and serve as intermediate hosts in the life cycles of metazoan parasites, such as, for example, trematodes and nematodes (Bojko & Ovcharenko, 2019). Infestations of Baikal endemic amphipods with nematodes, cestodes, acanthocephala and other metazoans were reported (Timoshkin et al., 2001). However, the sampled animals appeared all healthy and macroscopically showed no infestations with metazoans. Although the presence of metazoan symbionts, commensals, and parasites cannot be excluded, the overall tissue amount and thus the amount of extracted RNA from such organisms in relation to the extracted gammarid RNA can be considered as negligible. In the transcriptomes, the abundance of metazoan nongammarid transcripts was therefore assumed to only be minor in relation to gammarid transcripts. Metazoan sequences in the transcriptomes were thus generally assumed to be from the gammarids, even if assigned to other taxonomic groups.

The transcriptome of E. verrucosus muscle tissue served as reference presumably free of any nongammarid transcripts. From 29,083 contigs obtained from de novo assembled muscle tissue mRNA sequencing reads, 87.2% were assigned to metazoan species, most of the other contigs to bacteria and archaea and a small proportion to unicellular eukaryotes, plants, and fungi (Figure S2B). It is assumed that the prokaryotic sequences in the muscle tissue transcriptome are from bacteria contamination that occurred during dissection.

Upon filtering, the numbers of contigs assigned to G. lacustris, E. verrucosus and E. cyaneus were 61,366, 54,473 and 43,782, respectively (Table 2).

Functional annotation of transcripts in the assemblies was performed through orthology assignment; 9,134, 5,601, and 6,715 genes were annotated for G. lacustris, E. cyaneus, and E. verrucosus, respectively.

Transcriptomes of individuals from the 1.5°C exposure were examined for significantly DE genes (Benjamini-Hochberg FDR < 0.05; throughout the study unless otherwise stated) versus those from the 12°C exposure. Numbers of DE genes with >4-fold changes were only 76 and 16 in E. verrucosus and E. cyaneus, respectively, but 1,251 in G. lacustris, indicating a more pronounced gene response in this species (Figure 3). Accordingly, sample-to-sample distance analysis showed more pronounced differences between G. lacustris individuals from the 1.5°C and 12°C exposures than for the Eulimnogammarus species (Figure S3).

3.4 | DE genes in E. verrucosus

In E. verrucosus, individuals exposed to 1.5°C, 63 genes were upregulated (≥2-fold change) compared to individuals from the 12°C treatment; for 44 genes, expression changes were ≥4-fold. Downregulation was seen for 56 genes in individuals from 1.5°C versus 12°C treatments; expression decreases were ≥4-fold for 32 genes (Figure 3). The heatmap in Figure 4a shows the 26 transcripts with the most pronounced DE (≥16-fold change): upregulated genes at 1.5°C encoded a component of the ASCC complex involved in DNA damage repair (ASCC2), THO complex subunit ALYREF involved in nuclear export of mRNA, chromodomain protein Chro, cilium- and flagellum-specific protein CFAP20 that plays a role in axonemal structure organization and motility; downregulated

| Species     | Before filtration | After filtration | Annotated contigs | Filtered contigs assigned to Metazoa |
|-------------|------------------|------------------|-------------------|-------------------------------------|
| G. lacustris| 712,077          | 470,734          | 138,990           | 61,366                              |
| E. verrucosus| 833,922          | 590,166          | 187,714           | 54,473                              |
| E. cyaneus  | 603,270          | 371,250          | 99,627            | 43,782                              |

TABLE 2 Numbers of filtered contigs after assembly, removal of redundant transcripts and misassembled contigs, annotation, and taxonomic filtration
transcripts encoded proteins involved in signal transduction (annexin ANXA13, ELMO domain-containing proteins ELMOD2 and ELMOD3, and a multifunctional protein Unc119 involved in signal transduction in immune cells), in protein assembly and degradation (heat shock protein Hsc70-5, protein disulphide-isomerase P4HB, derlin-2 DERL2, 26S proteasome non-ATPase regulatory subunit PSMD9, and deubiquitinating enzyme Josephin-1 JOSD1), and in mitotic and meiotic division (Aurora kinases A and C AURKA and AURKC, meiotic nuclear division protein MND1).

Gene set enrichment analysis of functionally annotated DE genes in E. verrucosus at 1.5°C versus 12°C (Figure 4 and Figure S4A) revealed the four most upregulated GO terms: gene expression (GO:0010467), RNA processing (GO:0006396), mRNA metabolic process (GO:0016071), and protein-containing complex subunit organization (GO:0043933) (Figure 4c). In addition, we identified GO terms related to reproduction: embryo development (GO:0009790) and fertilization (GO:1901565) (Figure 4e). Furthermore, genes involved in other processes were found to be more highly expressed: development-related genes multiple epidermal growth factor-like domains protein (MEGF8) and DALR anticodon binding domain-containing protein (DALRD3); moreover, transcripts encoding vitellogenin (Vg) and eukaryotic initiation factor 4A-1 (EIF4A1), known to untangle RNA secondary structures in the 5'-UTR of mRNAs (Figure 4b).

The four most abundant GO terms in gene set enrichment analysis (Figure 4 and Figure S4B) in E. cyaneus from the 1.5°C treatment were: regulation of cellular process (GO:000796), response to stimulus (GO:0050896), homeostatic process (GO:0042592), and organonitrogen compound catabolic process (GO:1901565) (Figure 4e). Particularly, the GO term homeostatic process may comprise genes associated with cold adaptation mechanisms (Figure 4f), such as procathepsin H (CTSH) related to protein degradation, ubiquitin-like modifier-activating enzyme (Uba1), RB1-inducible coiled-coil protein (RB1CC1) involved in autophagy, and V-type proton ATPase subunit B (ATP6V1B2), which is found in the membranes of endosomes and lysosomes. In addition, this GO term includes acyl-CoA desaturase (SCD) transcript encoding an enzyme known to incorporate double bonds in acyl chains of polyunsaturated fatty acids to maintain membrane fluidity at low temperatures.

3.5 | DE genes in E. cyaneus

From the studied species, E. cyaneus from the 1.5°C versus 12°C treatments showed the smallest number of DE genes. Expression was >2-fold increased for 29 genes, among which 11 genes were >4-fold upregulated. Expression levels of 43 genes were decreased >2-fold and of five genes >4-fold (Figure 3). Upregulation in 1.5°C exposed animals was most pronounced for genes associated with mitochondrial function: iron-sulphur protein NUBPL, essential for the assembly of the NADH dehydrogenase (complex I); Mpv17-like protein 2 (MPV17L2), required for the assembly and stability of the mitochondrial ribosome; the beta chain of mitochondrial propionyl-CoA carboxylase (PCCB) involved in the catabolism of odd chain fatty acids and branched-chain amino acids; and histidine ammonia-lyase (HAL), a component of the histidine degradation pathway that ends up by α-ketoglutarate production, a key molecule in the Krebs cycle (Figure 4b). Furthermore, genes involved in other processes were found to be more highly expressed: development-related genes multiple epidermal growth factor-like domains protein (MEGF8) and DALR anticodon binding domain-containing protein (DALRD3); moreover, transcripts encoding vitellogenin (Vg) and eukaryotic initiation factor 4A-1 (EIF4A1), known to untangle RNA secondary structures in the 5'-UTR of mRNAs (Figure 4b).

3.6 | DE genes in G. lacustris

In G. lacustris, 872 DE transcripts were >2-fold upregulated in the 1.5°C versus the 12°C treatment; expression levels of 763 DE transcripts were 2- to 4-fold and of 109 DE transcripts >4-fold increased. Downregulation was seen for 1638 DE genes (>2-fold change), comprising 496 DE genes with 2- to 4-fold and 1142 DE genes with >4-fold decrease in expression (Figure 3). The number of downregulated genes and the degree of gene expression changes indicate a response of G. lacustris to low temperatures resulting in generally decreased gene expression activity in this species.

Gene set enrichment analysis with functionally annotated DE genes was performed to identify overrepresented gene ontology (GO) terms in G. lacustris upon exposure to 1.5°C. Compared to animals exposed to 12°C, G. lacustris from the 1.5°C treatment showed upregulation of genes from biological process terms associated with events preceding translation: ribosome biogenesis (GO:0042254),
(a) *E. verrucosus*

![Gene expression heatmap for *E. verrucosus*.

(b) *E. cyaneus*

![Gene expression heatmap for *E. cyaneus*.

(c) *E. verrucosus*

- mRNA metabolic process
- RNA processing
- Protein-containing complex subunit organization
- Ribonucleoprotein complex biogenesis
- ncRNA metabolic process
- Embryo development
- DNA metabolic process
- Regulation of gene expression, epigenetic
- 'De novo' protein folding
- Regulation of DNA recombination
- Fertilization
- Establishment of chromosome localization
- DNA replication

(d) *E. cyaneus*

- Response to stimulus
- Organonitrogen compound catabolic process
- Homeostatic process
- Regulation of response to stress
- Positive regulation of catalytic activity
- Gland development
- Protein catabolic process
- Regulation of organelle organization
- Regulation of cell cycle process
- Innate immune response
- Gland morphogenesis
- Positive regulation of proteolysis
- Pattern specification process
- Modification-dependent macromolecule catabolic process
- Chaperone-mediated protein folding
- RNA splicing
- Regulation of body fluid levels

(e) *E. cyaneus*

- SLC25A2
- CTSH
- Ubil
- ATP5B1B
- SAA1
- SDC
- FEN1
- PTGES3
- HAL
- RB11C1

(f) *E. cyaneus*

- SLC25A2
- CTSH
- Ubil
- ATP5B1B
- SAA1
- SDC
- FEN1
- PTGES3
- HAL
- RB11C1

Number of genes:
- 3
- 33
- 42
- 62
RNA metabolic process (GO:0016070), mRNA processing (GO:0006396), and gene expression (GO:0010467) (Figure 5a).

Among the transcripts included in these GO terms, those with the most pronounced changes (>4-fold changes) were transcripts of the small and large ribosomal subunit proteins, Rpl23, Rps27L, Rps11, Rps27, and nucleolar GTP-binding protein (GNL2), required for the nuclear export of pre-60S ribosomal subunits; genes encoding RBM8A, SNRPD2, and HNRNPL, involved in splicing; genes of mRNA processing proteins: a subunit of the deadenylase complex PAN3, DEAD-box RNA helicase DDX5, and mRNA cap guanine-N7 methyltransferase RNMT (Figure 5b). Genes assigned to the KEGG pathway ribosome biogenesis (ko03008) were accordingly found to be upregulated in 1.5°C-exposed G. lacustris (Figure S5).

Furthermore, the GO term cellular response to cold (GO:0070417) was overrepresented (Figure 5a), comprising transcripts encoding a member of the antioxidant system, peroxiredoxin (PRDX4), a subunit of the deubiquitinating enzyme BRISC (FAM175B) and the E3 ubiquitin-protein ligase (RFFL).

Certain DEAD-box RNA helicases were previously shown to be enriched in prokaryotes and eukaryotes at cold stress (Gracey et al., 2004; Guan et al., 2013; Hunger et al., 2006; Yang et al., 2014); transcriptomes of G. lacustris from the 1.5°C treatment were therefore examined for DE DEAD-box RNA helicase transcripts. Overall, 19 respective genes showing DE were found (FDR <0.001, Figure 6). Downregulation at 1.5°C was seen for DDX10, DDX60, DDX23, DDX46, DDX21, DDX24, DDX3X, and DDX6; upregulation for DDX17, DDX5, DDX18, DDX54, DDX39A, DDX27, DDX20, DDX47, DDX52, DDX51 and DDX55, which thus may comprise cold-inducible RNA helicases in G. lacustris.

Enrichment of the KEGG pathway mitogen-activated protein kinase (MAPK) signalling pathway (ko04010) in G. lacustris from the 1.5°C treatment reveals a number of upregulated genes encoding mitogen- and stress-activated kinases 1 and 2 (Msk1/2), mitogen-activated protein kinases 1, 7, and 13 (MEKK1, TAK, LZK), and related target proteins activating transcription factor ATF2 and ETS transcription factor Elk1. Furthermore, genes encoding antagonizers of the MAPK cascade, phosphatases MKP and PP2CB, were upregulated and mitogen-activated protein kinases 1 and 14 (ERK and p38) found to be downregulated (Figure S6).

Genes downregulated in G. lacustris at 1.5°C were associated to GO terms cellular component assembly (GO:0022607), sexual reproduction (GO:0019953), immune system process (GO:0002376), cell cycle (GO:0007049), cell division (GO:0051301), nucleotide metabolic process (GO:0009117), and generation of precursor metabolites and energy (GO:0006091) (Figure S5).

In G. lacustris from the 1.5°C treatment downregulated genes were enriched in GO terms related to cilia motility and cell trafficking: cellular component assembly (GO:0022607), microtubule-based process (GO:0007017), cell projection organization (GO:0030030), cilium or flagellum-dependent cell motility (GO:0001539), and sperm motility (GO:0097722) (Figure 5c,d). Several of those genes encode heavy and light chains of axonemal dynein proteins (DNAH3, DNAH5, DNAH6, DNAH9, DNAH12, DNAL1), dynein regulatory proteins (subunit of dynein regulatory complex TCTE1, axonemal dynein assembly factor DNAAF1), and kinesins (Kif17, Kif28p, Kif3c). Furthermore, DE genes were identified that are associated with cilia development and operation, such as doublecortin Dcduc2 and intraflagellar transport protein IFT52 involved in ciliogenesis, adenylyl kinase AK7 maintained ciliary structure and function, component of ciliary axonemes Spag17, and cilia- and flagella-associated protein CFA61 (Figure 5d). The enrichment of regulation of actin cytoskeleton (ko04810) pathway members revealed a significant group of genes downregulated at 1.5°C versus 12°C, mostly in G. lacustris but not in the Eulimnogammarus species (Figure S7).

4 | DISCUSSION

This study investigated the transcriptomes of amphipods from the Lake Baikal area exposed to the mean water temperatures during summer (12°C) and winter (1.5°C) (Figure 1). In winter, the species show marked differences in their metabolic activities and behaviours. We aimed to identify the transcriptomic expression patterns underlying the different species-specific physiological activities at the winter-specific cold temperature regimes. These comprise strongly reduced metabolic and locomotor activity in G. lacustris but the maintenance of high activities in the Baikal-endemic Eulimnogammarus species, with reproduction taking place in E. verrucosus. To our knowledge, this is the first study on cold acclimation responses on the transcriptomic level in amphipods.

For G. lacustris, the general characteristics of the transcriptome were in line with the metabolic and behavioural activity profile at low water temperatures: the fact that DE genes were predominantly downregulated is correspondent to metabolic depression occurring in G. lacustris at temperatures close to 0°C. Upon exposure to 1.5°C, the number of DE genes in the Eulimnogammarus species, showing high metabolic activity at cold temperatures, was considerably smaller than in G. lacustris. The observed low responsiveness indicates low sensitivity of the endemic species to low temperature.
Cold exposure induces the onset of hibernation in *G. lacustris*

Gammarus lacustris from the 1.5°C treatment showed a pronounced transcriptomic response, along with low locomotor and feeding activity. At temperatures close to 0°C, this species drastically decreases its metabolic activity and falls into torpor (Axenov-Gribanov et al., 2016; Karanova & Andreev, 2010). A gradual decrease in temperature from 10.5°C to 1.5°C at a rate of −0.5°C per day leads to majorly decreased metabolic rates that deviated from those that would be expected according to the $Q^{10}$ rule ($Q_{10} = 6$; Hegarty, 1973; Jakob et al., personal communication). It may thus be assumed that the strong transcriptomic response of *G. lacustris* is driven by its physiological state rather than being a direct effect of low temperature; the transcriptomic response pattern may thus be related to the species’ high seasonal changes in locomotor activity. The strategy of *G. lacustris* to cope with seasonal temperature changes is based on profound changes in locomotor and metabolic activity. In summer, the species’ physiological activity level is comparatively high: *G. lacustris* was found to have more powerful mitochondria than the studied *Eulimnogammarus* species (Jakob et al., 2021; Vereshchagina et al., 2021). However, high-performing mitochondria are energetically more costly; correspondingly, at the low temperatures in winter *G. lacustris* decreases its metabolic activity levels to a high degree to prevent energy depletion. The three overarching tasks, (1) finding food, (2) escaping predators, and (3) reproducing drive natural selection of the most beneficial locomotor activity (Visser 2007). In this context, various habitat-specific parameters in winter, such as oxygen levels, food availability, abundance of potential predators and the presence of overwintering habitats, enable but also require the diverging metabolic states of *G. lacustris* overwintering in a torpid stage and of the Baikal amphipod species maintaining high metabolic activity.

According to the dynamic energy budget model, organisms allocate available energy primarily to growth, activity, reproduction, and basal maintenance (Sokolova et al., 2012). In order to survive
in the cold during hibernation, energy is entirely allocated to basal maintenance (Wilsterman et al., 2021). Accordingly, we found a high proportion of suppressed genes related to activity, growth, and reproduction in cold-exposed G. lacustris. Moreover, cold exposure induced an enrichment in genes involved in the MAPK signalling pathway, which was shown to play an essential role in the onset of hibernation in vertebrates and invertebrates (Biggar et al., 2015; Childers et al., 2019; Fujiwara et al., 2006; MacDonald & Storey, 2005; Michaelidis et al., 2009; Tessier et al., 2017; Wijenayake et al., 2018; Zhang & Storey, 2017). The observed low locomotor activity of cold-exposed G. lacustris might be due to decreased expression of genes related to the regulation of the actin cytoskeleton pathway, as cytoskeleton stabilization was shown to play a central role for locomotor activity in the cold for a wide variety of organisms (Kim & Denlinger, 2009; Königer & Grath, 2018; Lee et al., 1998). The detected decreased expression levels of transcripts associated here to cilia motility-related GO terms (Figure 4d) may concern so-called sensilla, cilia on sensory cells of crustaceans, functioning as chemoreceptors (Hessler & Elofsson, 2013) or mechanoreceptors (lacinia mobilis, a hair sensillum on mandibles; Geisler & Melzer, 2013). There is no epithelium with motile cilia reported for the amphipods. In this context, it is noticeable that transcripts encoding axonemal dyneins were detected in the G. lacustris transcriptome (Figure 5d).

Upon exposure to low temperature, a number of genes showed enhanced expression. Up-regulation of protein expression can compensate for a temperature-related decrease in enzymatic activity (Gracey et al., 2004; Li et al., 2019; Sonna et al., 2002; Wang et al., 2013). Increased expression of genes involved in ribosome biogenesis and mRNA processing may be related additionally to the compensation of reduced enzyme activity rates at low temperatures as the respective gene products stabilize the protein machinery for basal maintenance. Previously, the upregulation of ribosomal biogenesis in ectotherms in response to cold was found (Chen et al., 2008; Long et al., 2013). Short-term cold exposure of porcelain crabs induces expression of genes encoding proteins involved in DNA and RNA binding, their modification, and in the regulation of transcription and translation (Ronges et al., 2012). Upregulation of DEAD-box RNA-helicases in gammarids from the 1.5°C exposure (Figure 5) could be related to reduced integrity of nucleic acids due to low temperature (Tinoco & Bustamante, 1999). Thus, DEAD-box RNA helicases act as enzymes unwinding nucleic acid strands, mitigating stress effects on nucleic acid integrity (Yadav & Tuteja, 2019).

4.2 | Baikal endemic amphipods — maintenance of high metabolic activity in the cold

When organisms were long-term acclimated to certain conditions, the magnitude of their transcriptomic response to a stress factor is suggested to reflect the differences in whole animal performance in stress versus no-stress conditions (Windisch et al., 2014). A weak long-term response to thermal changes in an organism thus indicates that an exposure temperature was within a species’ thermal tolerance window, within which acclimation had taken place. Along those lines, the comparatively weak transcriptomic reaction of the studied Baikal amphipod species to 1.5°C exposure, as well as their high locomotor and feeding activities at this temperature indicate that 1.5°C is within their thermal window for survival and activity. However, cold acclimation can also occur on the levels of cellular organization that do not become evident on the transcriptome level, concerning, for example, post-translational events and protein structure.

Although the two Eulimnogammarus species showed little transcriptomic changes upon cold exposure, their transcriptomic profiles show differences related to the ecology of the species. Reproduction of E. verrucosus starts in November to January in conditions of stable cold temperatures in the littoral of Lake Baikal (Gavrilov, 1949). Accordingly, we found an increase in the expression activity of genes related to translation, fertilization and embryonic development, probably indicating the beginning of reproductive processes in E. verrucosus (Figure 6Cc). The physiological performance of the cold-loving E. verrucosus indicated slight heat stress when exposed to 12°C, this temperature being beyond the upper pejus (Latin for "getting worse") temperature of E. verrucosus (Jakob et al., 2016). Upregulation of cellular and metabolic stress response genes in E. verrucosus from the 12°C treatment could therefore be anticipated and, indeed, higher levels of transcripts of Hsps, other molecular chaperones and protein degrading enzymes were found in animals from this treatment. Although this indicates a cellular stress response in this species at 12°C, the comparatively small number of DE genes indicate a rather weak stress response.

In E. cyaneus acclimated to 1.5°C, several genes related to mitochondria functioning were activated, a common feature of cold compensation observed both in cold-adapted species and cold-acclimated individuals. Thus, cold-adapted species show an increase in mitochondrial enzyme capacities and in mitochondria density (Fangue et al., 2009; Lucassen et al., 2006; Pörtner, 2002). Eulimnogammarus cyaneus was shown to have lower mass-specific metabolic rates than G. lacustris and lower cytochrome c oxidase/citrate synthase (COX/CS) enzyme activity ratios (Jakob et al., 2021). This may indicate high mitochondrial densities with relatively low capacities of individual mitochondria (compared to G. lacustris), which is a feature of metabolically active cold-adapted animals (Clarke & Johnston, 1999).

In E. cyaneus, we also detected upregulation of genes involved in homeostatic processes, including protein degradation. Again, this may indicate some level of cold compensation of this basal process, but may also suggest an increase in protein misfolding events in a low energy (i.e., low temperature) environment. Similarly, activation of the proteasome degradation pathway was found in Antarctic fish upon long-term cold exposure (Windisch et al., 2014). Moreover, enhanced expression of acyl-CoA desaturase transcript (SCD; Figure 6f) at 1.5°C may reflect the compensation of decreased membrane fluidity (Kates et al., 1984) in the frame of homeoviscous adaptation (Hazel, 1995). Strikingly, indications for homeoviscous adaptation processes at low temperature were only obtained for E. cyaneus, not for the other species.
The gene encoding Vg, a precursor of the egg yolk protein, was significantly upregulated in *E. cyaneus* from the 1.5°C treatment (Figure 4b), which may not be expected as *E. cyaneus* is a summer-reproducing species. However, the Vg encoding gene was also shown to be increasingly expressed in bee larvae exposed to cold stress (Ramirez et al., 2017), suggesting that Vg might also play a protective role in stress conditions.

Both studied Baikal species show reduced growth rates in winter (Gavrilov, 1949; Govorukhina, 2005). Due to the prolonged period of embryonic development of about 6–7 months in *E. verrucosus*, this species needs to allocate a major share of the available energy to reproduction in the winter months (Gavrilov, 1949; Govorukhina, 2005). In contrast, *E. cyaneus* starts reproducing in May; embryos develop in about one month during the summer (Govorukhina, 2005), and the reduced growth rates in cold conditions are probably due to increased energy demands for maintaining homeostasis.

The transcriptomic responses of the two Baikal endemic *Eulimnogammarus* species to the low temperature treatment were remarkably little. This may be due to a particularly high tolerance of the species’ metabolic and physiological functions within the studied thermal range and certain post-transcriptional and translational processes, including those of protein modification and protein stability/turnover. This may enable the species to cope with strongly fluctuating water temperatures that are characteristic for the species’ habitat. Thus, water temperatures frequently change rapidly by up to 0.6°C/h over a range of up to 10°C during the summer months (Figure 1). By having evolved eurytolerant proteins with stable ligand-binding capacity over the entire range of the encountered habitat temperatures (Somero & Low, 1977) endemic inhabitants of the littoral of the unique Baikal ecosystem may have acquired a competitive advantage over ubiquitous Holarctic species, such as *G. lacustris*, that may be more sensitive to rapid temperature changes. This may thus be a key parameter of the “species immiscibility barrier” (Timoshkin, 2001) preventing Holarctic, non-Baikal species from establishing stable populations in regions with typical Lake Baikal fauna.

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**AUTHOR CONTRIBUTIONS**

Kseniya Vereshchagina, Magnus Lucassen, Lena Jakob, Daria Bedulina and Maxim Timofeyev developed the concept and design of the experiment. Kseniya Vereshchagina and Elizaveta Kondrateva performed the exposure experiments and sampled the amphipods. Polina Lipaeva performed RNA extractions and prepared the samples for sequencing. Polina Lipaeva analysed the data with the help of Polina Drozdova and Peter Stadler. Polina Lipaeva and Till Luckenbach wrote the manuscript, which was revised by Lena Jakob, Magnus Lucassen, Daria Bedulina, Polina Drozdova, Peter Stadler and Kseniya Vereshchagina. All authors read and approved the manuscript.

**DATA AVAILABILITY STATEMENT**

The data sets generated during the current study have been submitted to the NCBI database under the BioProject PRJNA660769 (Bedulina et al., 2020a), including the raw RNA sequencing data in the SRA database. The transcriptome assemblies have been submitted to the GenBank database under the accession numbers GIUS00000000, GIUW00000000, GIUX00000000, GJDV00000000 (Lipaeva et al., 2021a, 2021b, 2021c, 2021d). The differential expression data are available in the GEO repository under the accession number GSE157288 (Bedulina et al., 2020b). In-house scripts for data analysis are available at https://github.com/PolinaLip/Amphipods_ColdAdaptation_RNA.

**ORCID**

Polina Lipaeva https://orcid.org/0000-0002-8416-8323
Polina Drozdova https://orcid.org/0000-0003-3955-6105
Maxim Timofeyev https://orcid.org/0000-0002-5250-6818

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