Temperature-dependent life history and transcriptomic responses in heat-tolerant versus heat-sensitive Brachionus rotifers

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Abstract

A species' response to thermal stress is an essential physiological trait that can determine occurrence and temporal succession in nature, including response to climate change. Environmental temperature affects zooplankton performance by altering life-spans and population growth rates, but the molecular mechanisms underlying these alterations are largely unknown. To compare temperature-related demography, we performed cross-temperature life-table experiments in closely related heat-tolerant and heat-sensitive Brachionus rotifer species that occur in sympatry. Within these same populations, we examined the genetic basis of physiological variation by comparing gene expression across increasing temperatures. We found significant cross-species and cross-temperature differences in heat response, with the heat-sensitive species adopting a strategy of high survival and low population growth, while the heat-tolerant followed an opposite strategy. Comparative transcriptomic analyses revealed both shared and opposing responses to heat. Most notably, expression of heat shock proteins (hsps) is strikingly different in the two species. In both species, hsp responses mirrored differences in population growth rates, showing that hsp genes are likely a key component of a species' adaptation to different temperatures. Temperature induction caused opposing patterns of expression in further functional categories such as energy, carbohydrate and lipid metabolism, and in genes related to ribosomal proteins. In the heat-sensitive species, elevated temperatures caused up-regulation of genes related to induction of meiotic division as well as genes responsible for post-translational histone modifications. This work demonstrates the sweeping reorganizations of biological functions that accompany temperature adaptation in these two species and reveals potential molecular mechanisms that might be activated for adaptation to global warming.

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histone modifications. This work demonstrates the sweeping reorganizations of biological functions that accompany temperature adaptation in these two species and reveals potential molecular mechanisms that might be activated for adaptation to global warming.

Keywords: *Brachionus calyciflorus* , *Brachionus fernandoi* , thermal adaptation, RNA-seq, heat-stress

INTRODUCTION

On a global scale, a species' occurrence is related to its tolerance of a particular range of environmental parameters such as temperature, salinity and precipitation. In aquatic ecosystems, temperature has a profound impact on an organism's survival and performance, and can affect species abundance, spatio-temporal distribution, and habitat colonization (Paaijmans et al., 2013; Parmesan, 2006). There is great variation in the thermal tolerance among aquatic taxa. Many species can tolerate a broad range of temperature, while others have specific and narrow temperature limits (Cullum, 2008; Hershey, Lamberti, Chaloner & Northington, 2010). Importantly, this can impact temporal occurrence, and temperature-dependent seasonal succession has been well documented among genetically similar species that might have evolved species-specific temperature specializations (Papakostas, Michaloudi, Triantafyllidis, Kappas, & Abatzopoulos, 2013; Wen, Xi, Zhang, Xue, & Xiang, 2016; Xiang et al., 2011a; Zhang et al., 2017). Therefore, understanding species thermal boundaries is essential for comprehending how species have adapted to their environment and how they may respond to climate change.

Zooplankton are important components of aquatic ecosystems, as they transfer organic compounds and energy from primary producers (e.g. phytoplankton) to higher trophic levels (Segers, 2008). Among zooplankton, monogonont rotifers are of particular interest because of their high, often cryptic, diversity, their frequent adaptation to specific environmental conditions, and their high dispersal capability (Fontaneto, Kaya, Herniou & Barraclough, 2009; Mills et al., 2017). Species complexes formerly assumed to be ubiquitous generalists have been found to comprise cryptic species adapted to specific ecological conditions regarding temperature, habitat type, or salinity (Gabaldón, Fontaneto, Carmona, Montero-Pau, & Serra, 2017). As their dispersal capabilities can be large, distribution and diversification seems less dependent on geographical barriers and historical factors, suggesting that ecological specialization is more likely to drive speciation (Gómez, Serra, Carvalho & Lunt, 2002; Mills et al., 2017; Serra & Fontaneto, 2017; Suatoni, Vicario, Rice, Snell & Caccone, 2006). As evidence of specialization, co-occurrence of differentially adapted, closely related species in a single locality is a common phenomenon in rotifers (Papakostas, Michaloudi, Triantafilidis, Kappas & Abatzopoulos, 2013; Xiang, Xi, Wen, & Ge, 2017; Zhang et al., 2017). In these cases, morphologically similar species might have evolved different ecological specialties to reduce competition over resources in space or time (Fontaneto, Giordani, Melone & Serra, 2007; Montero-Pau, Ramos-Rodríguez, Ciros-Pérez, Serra & Gómez, 2011; Serra & Fontaneto, 2017).

The best studied freshwater monogonont rotifer is the *Brachionus calyciflorus* species complex that has recently been resolved to four different species: *Brachionus calyciflorus* sensu stricto (s.s.),*Brachionus fernandoi* , *Brachionus dorcas,* and*Brachionus elevatus* , using integrative taxonomy (Michaloudi et al., 2018; Papakostas et al., 2016). The species of this complex exhibit temporal succession, and their occurrences have been related to temperature in several studies (Li, Niu, & Ma, 2010; Wen et al., 2016; Zhang et al., 2017). More specifically, temperature constraints were shown to affect the temporal occurrence and abundance of *B. calyciflorus* cryptic species in different habitats in China, with*B. fernandoi* occurring in winter and spring, and *B. calyciflorus s.s.* in summer (Xiang et al., 2017; Zhang et al., 2017). Comparative laboratory studies on heat tolerance between *B. calyciflorus* s.s. and *B. fernandoi* have shown higher heat-tolerance of the former, thus confirming that temperature tolerance likely plays a role in their temporal distribution (Paraskevopoulou, Tiedemann & Weithoff, 2018).

Regulation of gene expression is an essential mechanism underlying phenotypic plasticity (Wray et al., 2003). As selection acts on expression, transcriptome data are particularly useful in revealing genes contributing to phenotypic plasticity. Differences in gene expression might have been evolved via divergent selection as a consequence of ecological differentiation (Romero, Ruvinsky, & Gilad, 2012). Aquatic taxa use a variety

of physiological mechanism to cope with temperature changes. Transcription studies on heat shock response have often focused on genes encoding for heat shock proteins (*hsps*). Heat shock proteins are divided into several groups (families) of different molecular weights (kDa): e.g.*hsp90, hspP70, hsp60, hsp40* , and small proteins. *Hsp70s* , in combination with other proteins, play a vital role in stress tolerance and survival under adverse conditions. They reduce accumulation of peptide aggregates and promote the correct folding of newly synthesized proteins (Mayer & Bukau, 2005). *Hsp90s* play also a major role in stress tolerance, mainly by removing incorrectly folded proteins. Furthermore, they regulate the activity of other proteins (e.g., kinases) and stabilize the cytoskeleton (Csermely, Schnaider, Soti, Prohászka, & Nardai, 1998). Induction of *hsp*genes is an evolutionary old and conserved mechanism, and is described from prokaryotes to higher eukaryotes (Feder & Hofmann, 1999). However, the specific genes involved and the conditions of induction vary among taxa (Parsell & Lindquist, 1993). In rotifers, particularly *Brachionus* species, members of the*hsp70* and *hsp40* families increase heat shock survival, suggesting that there may be coordination among heat shock proteins in which *hsp40* works synergistically to regulate *hsp70* 's activity (as shown in *B. manjavacas* ; Smith, Burns, Shearer, & Snell, 2012).

To investigate the marked variation in thermal tolerance between two closely related species in the former *B. calyciflorus* species complex (Paraskevopoulou et al., 2018), we compared life-history demography and gene expression under mild to high temperature conditions. We use life-table experiments to examine survival, fecundity, and population growth rate differences between the two species. We collect transcriptomic data (RNA-seq) to examine the genetic basis of physiological response and its difference between the two species. Identifying the mechanisms involved in thermal tolerance and investigating their variation among heat-tolerant and heat-sensitive species is important to understand how physiology determines species' temporal distribution, and how this might be affected by different scenarios of climate change.

MATERIALS AND METHODS

2.1 Rotifer culture and life table experimental conditions

Lab-reared, asexually reproducing clones of the heat-tolerant *B. calyciflorus s.s.* (clone IGB; CT_{max} =43.18°C) and the heat-sensitive *B. fernandoi* (clone A10; CT_{max} =38.49 °C) were selected for our experiments. Both clones originate from Northern Germany and were reared under laboratory conditions for more than 10 years. Species identity was previously confirmed by amplifying a portion of the ITS1 genetic marker (Paraskevopoulou et al., 2018). Stock cultures were maintained as batch cultures in glass flasks containing WC medium (Guillard & Lorenzen, 1972) at 20 °C under a 16:8 light:dark photoperiod. A food combination of two algae species,*Monoraphidium minutum* (Culture collection Göttingen, strain SAG-243-1) and *Cryptomonas* sp. (Culture collection Göttingen, strain SAG-26-80), was provided weekly.

Before starting the life table experiments, cultures were exposed to a period of gradual acclimatization by increasing the temperature 2 °C every 2 days until reaching the experimental temperature. Because of this, the acclimation period varied among cultures, with the longest adaptation period (2 weeks) for the highest temperature (32 °C). After reaching the experimental temperature, we maintained the rotifer cultures for one week (at least two to four generations) before starting the experiment to allow for acclimation, and to reduce potential maternal effects. Food was supplied *ad libitum* daily. For*B. calyciflorus* s.s., experiments were conducted at four temperatures (20 °C, 23 °C, 26 °C, 32 °C), while for *B. fernandoi*three temperature assays (20 °C, 23 °C, 26 °C) were used. We tried several times to acclimatize *B. fernandoi* also to 32 °C, but high mortality always led to culture collapse before the initiation of the experiment.

The experiments basically followed the procedure from Weithoff (2004, 2007): single females bearing a subitaneous, asexual egg were isolated from the culture, placed in a microtitre well, and inspected thoroughly for hatched neonates. Life-table experiments were started by introducing one neonate into a new well and adding 1ml of algal suspension composed of *Monoraphidium minutum* (5 x 10^5 cells/ml) and *Cryptomonas* sp. (2 x 10^4 cells/ml), to avoid food limitation. For each temperature and species, at least 24 individuals were recorded. Survival and reproduction were recorded every 12 h and any newly hatched neonates were removed. Every 24h the maternal individuals were transferred into a new well with fresh food suspension.

The experiment was conducted in the dark and continued until all individuals of each cohort died.

2.2 Computations and statistical analysis

The variables of average life span (survival), age-specific survival (lx) and age-specific fecundity (mx) were estimated for each species and temperature assay (x was defined as the age interval in days, lx as the proportion of surviving individuals at the beginning of the age interval and mx as the number of offspring produced per female alive from the start until the end of any age interval (Poole, 1974). The intrinsic rate of population increase (r) was also estimated from these data using Lotka's equation (Lotka, 1907):

$r=[?]e^{-rx}$ $*lx\ mx = 1$

Kaplan-Meier survival curves were calculated to compare survival across species and across temperatures. Differences between survival functions were analyzed pairwise using a log-rank test. To further estimate the effect on lifespan and fecundity by species, temperature, and their interaction, we used generalized linear models (GLMs). We selected the best-fitting model based on the AIC criteria (Zuur, Leno, Walker, Saveliev, & Smith, 2009). To compare fecundity among the species and temperature treatments we used the non-parametric Kruskal–Wallis one-way analysis of variance. For pairwise comparisons the pairwise Wilcox test was used with a Bonferroni correction for the p-values. Non parametric tests were used for comparisons, as all of the assumptions to perform Analysis of Variance (ANOVA; normal distribution of the data, normal distribution of the residuals, and homoscedasticity) were violated. To compare the intrinsic rate of increase (r), the 95% confidence interval was estimated via bootstrapping with 199 iterations (Weithoff & Wacker, 2007) using an R script (personal communication Dr. Wacker). All statistical analyses were performed using R 3.4.1 (R Core Team).

2.3 Sample cultivation, collection, and RNA isolation

Samples for RNA-seq were first cultivated as batch cultures in 1L glass flasks containing WC medium at 20 degC under 16:8 light:dark photoperiod. The same food combination of two algae species that used in life-table experiments was provided *ad libitum* every two days for two weeks before the RNA-seq experiment. For each species and temperature, we sub-sampled the initial stock into new flasks to create four replicates of 200ml each, containing more than 1000 individuals. We placed the flasks into water-baths adjusted to the experimental temperature and heat-exposed the rotifers for 4 hours. Experimental temperatures were selected according to the life-table results in order to represent control (20 °C; temperature in which both clones were acclimated), mild heat treatment (23 °C for *B. fernandoi;* 26 °C for *B. calyciflorus* s.s.; intermediate temperature between control and high heat exposure), and high heat treatment (26 °C for *B. fernandoi* and 32 °C for *B. calyciflorus* s.s.; temperature in which the population growth rate starts to decline) (Figure 1). For specimen collection we filtered each replicate (200ml) through a 30µm sieve, re-suspended what remained on the filter in WC medium, and centrifuged it at 2000 ×g for 10 minutes to pellet phytoplankton and other debris, before transferring the rotifers (remaining in the supernatant) into 1ml of TRIzol®LS and storing them at -80°C until RNA extraction. For RNA extraction, we used four replicates from each temperature and species with the exception of *B. fernandoi*at mild heat, where two replicates were used. Each replicate, comprised approximately of 1000 individuals, reared in independent flasks of 200ml.

Samples in TRIzol®LS were homogenized using a Tissue Lyzer (4min, 50HZ) and were incubated overnight at room temperature. A total of 500µl of chloroform was added to each sample, and samples were centrifuged for 15 minutes at 4°C to facilitate phase separation. We then transferred the colorless, upper aqueous phase into an RNeasy® Mini Kit column (Qiagen, Germany) and proceeded to RNA precipitation according to the manufacturer's instructions. A double elution step was performed with 20µl RNase-free water each. Total RNA concentration was estimated using a NanoDrop 1000 spectrometer (ThermoFischer Scientific, Germany). Quality of total RNA was examined using Agilent Bioanalyzer 2100 (Agilent Technologies, USA).

For transcriptomic library preparation, mRNA was enriched from 3µg of total RNA with poly (A) capture using NEXTflex Poly (A) Beads, and strand-specific libraries were built using NEXTflex Rapid Illumina

Directional RNA-Seq Library Prep Kit (Bioo Scientific, USA) according to manufacturer's instructions. Final elution was performed in 16µl of elution buffer and a PCR amplification of 14 cycles was performed. Libraries were quantified using Qubit dsDNA HS Assay Kit (Invitrogen, Germany) and quality control was performed using Agilent Bioanalyzer 2100 (Agilent Technologies, USA).

2.4 Transcriptome sequencing, assembly and annotation

Libraries were sequenced as 150 bp, paired-end (PE) reads using an Illumina HiSeq4000 sequencing system, performed by Novogene (Hong Kong, China). Raw data have been deposited in the NCBI Short Read Archive under the accessions numbers (SRA: SRR10426055-76). Adapter sequences were trimmed and low quality reads were filtered using a 5 bp sliding window with a mean quality threshold of 20 and minimum read length of 36 bp using Trimmomatic v0.36 (Bolger, Lohse, & Usadel, 2011). Read quality (before and after quality-filtering) was assessed using FastQC v0.11.5 (Andrews, 2010).

Processed reads were assembled *de novo* with Trinity v.2.5.1 (Grabherr et al., 2011). Separate reference transcriptomes were created for *B. calyciflorus* s.s. and *B. fernandoi* , using all reads generated for the respective species. To filter possible contamination, we used a custom perl script which, using the blastn algorithm (ncbi-blast-2.6.0; Camacho et al., 2015), assigns all contigs either to a local algae database (*Monoraphidium minutum* ,*Chlamydomonas reinhardtii* and, *Cryptomonas* sp) or to the respective *B. calyciflorus s.s.* genome assembly (Kim et al., 2018). Transcripts were only assigned as of rotifer origin when the top hit was to the *B. calyciflorus* s.s. genome and the bit-score gain over matches to the next species was >100. The same custom script was further used to remove ribosomal RNA reads by performing a blastn search to a local database consisting of 18S and 28S sequences of*Brachionus* species downloaded from NCBI. We did not utilize the*B. calyciflorus s.s.* reference genome for the analyses presented here, as this would introduce a bias, since the *B. fernandoi*reads would not map as well to the reference as those from *B. calyciflorus* s.s..

2.5 Identification of differentially expressed genes and pathways

To quantify expression we ran the "align_and_estimate_abundance.pl" script in Trinity v.2.5.1 (Grabherr et al., 2011) software incorporating the RSEM program (Li & Dwey, 2011). Gene-level quantification estimates produced by RSEM were imported into R/Bioconductor with the tximport package. The tximport package produces count matrices from gene-level quantification files with effective gene length taken into account (Soneson, Love, & Robinson, 2016). To detect differential gene expression, we passed the estimated count matrices from tximport to DESeq2 (Love, Huber, & Anders, 2014) and analyzed the two species separately. To build the model for the differential expression analysis we removed low count genes (<10) and genes that were not present in at least 2 replicates. To examine intra-species temperature specific expression pattern in *B. calyciflorus* s.s., we performed pairwise contrasts within the model in the following combinations: 20 *vs.*26 °C (control *vs.* mild heat), 20 *vs.* 32°C (control *vs.* high heat), and 26 *vs.*32 °C (mild *vs.* high heat) (Figure 1). For*B. fernandoi* similarly we performed pairwise comparisons in the following combinations: 20 *vs.* 23 °C (control*vs.* mild heat), 20 *vs.* 26 °C (control*vs.* high heat), and 23 *vs.* 26 °C (mild*vs.* high heat) (Figure 1). We used a false discovery rate (FDR) threshold of 0.05 to correct for multiple testing. All differentially expressed genes (DEGs) were annotated against the NCBI non-redundant (*nr*) database using blastx (e-value cutoff $1e^{-10}$). We assessed overall temperature-dependent patterns of expression by plotting a two-dimensional principal component analysis (PCA) of log-transformed counts for each species separately.

We further categorized differential expression at the gene-pathway level using the online Kyoto Encyclopedia of Genes and Genomes (KEGG) automatic server for KEGG pathway analysis (Moriya, Itoh, Okuda, Yoshizawa, & Kanehisa, 2007), which clusters genes based on their association in biochemical pathways. To estimate whole KEGG pathway expression, genes belonging to the same KEGG pathway were clustered together and a differential pathway expression analysis was performed. Pathways were considered differentially expressed at a false discovery rate (FDR) below 0.05. Analyses and visualization was performed using the "gage" (Luo et al., 2009), "clusterProfiler" (Yu, Wang, Han, & He, 2012), "pathview" (Luo, Weijun, Brouwer, & Cory, 2013), and "ggplot2" (Wickham, 2016) R packages (R Core Team).

To capture similar or contrasting patterns of expression between the two species, orthologous genes were

identified using Orthofinder (Emms & Kelly, 2015). From this, we compared expression of orthologous genes between *B. fernandoi* and *B. calyciflorous* using*Clust* (Abu-Jamous & Kelly, 2018). In *Clust,* gene clusters (groups) are identified that are consistently co-expressed (well-correlated) in both shared and contrasting patterns between species. Within this, we chose patterns that were biologically meaningful for further analysis. These groups/clusters were checked for functional enrichment in any KEGG pathways, using a Fisher Exact Test and correcting for false positives (FDR=0.05).

3. RESULTS

3.1 Life history responses to heat

The life history traits survival, fecundity and the resulting population growth rate of both species were strongly affected by temperature. For both species, survival was significantly reduced from control to mild and from mild to high heat (Figure 1A, all p-values available in Table S1). Cross-species survival comparisons revealed significant differences at 20 °C ($p<0.001$) and 23°C ($p=0.08$), with *B. fernandoi* surviving longer than *B. calyciflorus* s.s (Figure 1A, Table S1). Fecundity analysis with the K-W test revealed that both *B. calyciflorus*s.s. and *B. fernandoi* had a significant lower fecundity at the highest imposed heat (*B. calyciflorus* s.s.: 20°C *vs.* 32 °C, $p<0.001$; 23 °C *vs.* 32°C, $p=0.001$; 26 °C *vs.* 32°C, $p=0.002$; *B. fernandoi* : 20°C *vs.* 26 °C, $p<0.001$; 23 °C *vs.* 26°C, $p=0.004$, Figure S1A, Table S1). Across the two species, significant differences were observed at 26°C, in which the heat-tolerant *B. calyciflorus*s.s. had higher fecundity than the heat-sensitive *B. fernandoi*(Bcal26 *vs.* Bfer26, $p<0.001$, Figure S1A, Table S2). Generalized linear models revealed that both survival and fecundity were significantly dependent on temperature (GLM: survival, $p<0.001$; fecundity, $p<0.001$), species (GLM: survival, $p<0.001$; fecundity, $p=0.006$), and their interaction (GLM: survival, $p< 0.001$; fecundity, $p=0.001$) (Figure S1B, S1C).

The intrinsic rate of population increase (r) was above zero for both species, indicating a positive growth rate at all tested temperatures. However, r was always higher for the heat-tolerant*B. calyciflorus* s.s. than for the heat-sensitive *B. fernandoi* (Figure 2B). For *B. calyciflory*us s.s, $r$increased with the increase of temperature until its maximum at 26°C. In contrast, r remained constant in*B. fernandoi* from 20 °C to 23°C, and decreased at 26 °C.

3.2 Comparative transcriptomics

Sequencing of the *B. calyciflorus* s.s. and *B. fernandoi*transcriptomes generated 670,242,336 and 358,527,278 quality-filtered PE reads, respectively, with approximately equal numbers of reads among libraries (Table S3). Information about the *de novo* assemblies, assembled unigenes, and KEGG KO term assignment can be found in Table 1.

3.3 Cross-temperature differential gene expression

We first examined the expression data for temperature-dependent responses in each species by a two-dimensional plot of the first two principal components of a PCA. This analysis showed temperature-dependent separation of replicates for the heat-tolerant,*B. calyciflorus* s.s. This separation was less clear for the heat-sensitive species, in which replicates from 20 °C and 23 °C clustered together, while all samples treated at 26 °C formed a separate cluster (Figure S2). Overall, we found a greater number of differentially expressed genes (DEGs) in the heat-tolerant species than the heat-sensitive. In both species we captured a greater number of DEGs in pairwise comparison control*vs.* high heat (Figure 3A, 3B). However, in the heat-tolerant species, we captured a greater number of genes differentially expressed between control and mild heat (1268) than between mild and high heat (64). In contrast, in the heat-sensitive we found the opposite pattern (Figure 3A, 3B).

We focused on pairwise comparison mild *vs.* high heat in both species as we had evidence from population growth rate data that there is a strong effect of temperature in this transition. In *B. calyciflorus* s.s., among the 64 genes differentially expressed between mild (26 °C) and high (32 °C) heat, were genes encoding for RNA polymerases, histone proteins and transferases. The above genes showed different temperature induction with genes encoding for RNA polymerases and N-acetyltranferases being up-regulated at high heat. Genes

encoding for histones such as H2B family were either up- or down- regulated at high heat (Figure S3A). In heat-sensitive *B. fernandoi* , among the 310 differentially expressed between mild (23 °C) and high (26°C) heat, we found genes encoding for several histone methyltranferase proteins such as H3K4, H3K79, H4K20. All above genes were up-regulated with heat (Figure S3B).

We further focused on genes that are differentially expressed in all the three pairwise comparisons. These genes are of high importance as they are the most responsive to temperature presenting either up- or down-regulation along with temperature increase. In the heat tolerant*B calyciflorus* s.s., there were 23 genes differentially expressed in all pairwise comparisons. Among these, genes encoding for ribosomal proteins and glutathione S-transferase were up-regulated at lower temperatures, while proteases related genes were up-regulated at higher temperatures (Figure S4A). In the heat-sensitive *B. fernandoi,*there were two genes that were differentially expressed in all pairwise comparisons. These genes encode for E3 ubiquitin-ligase and a mediator of RNA polymerase were up-regulated at higher temperatures (Figure S4B).

3.4 Cross-temperature differentially expressed genes in Heat Shock Response

We examined patterns of expression in *hsp* genes to evaluate their specific contribution to the heat shock response (HSR) in both species. In both species, we found a significant down-regulation of heat shock protein genes at temperatures where population growth rate was maximized (Figure 1B, Figure 4A, 4B). In general, genes encoding for *hsps*had higher expression at the lowest temperature treatment (20°C) for the heat-tolerant, *B. calyciflorus* s.s. and at the highest temperature for the heat-sensitive, *B. fernandoi.* Genes encoding for *hsp* 27 and *hsp* 70 followed this pattern. Genes encoding for *hsp* 10, *hsp* 40, and*hsp* 60 were differentially expressed only in *B. calyciflorus* s.s. and followed the induction pattern of *hsp* 70. Genes encoding for *hsp90* beta were up-regulated under the highest imposed temperature regime in both species. In *B. calyciflorus*s.s., genes encoding for *hsp20* were also up-regulated under the highest imposed temperature (32 °C), following the induction pattern of *hsp90* beta (Figure 4A).

3.5 Cross-temperature differential KEGG pathway expression

To capture shared and contrasting patterns at the level of gene- pathways, we examined whole KEGG pathway expression. We compared whole KEGG pathway expression in two pairwise comparisons, control *vs.*mild heat and mild *vs.* high heat, because these comparisons represent a stepwise temperature induction. Overall, mild heat resulted in down-regulation of pathways related to genetic information processing in heat-tolerant, *B. calyciflorus* s.s., and up-regulation of metabolic related pathways in the heat-sensitive, *B. fernandoi* . More specifically, pathways "ribosome", "proteasome", and "oxidative phosphorylation" were down-regulated under mild heat exposure for *B. calyciflorus* s.s. and up-regulated for *B. fernandoi* (Figure 5A, Figure S5, S6). In general, high heat caused up-regulation of pathways related mainly to metabolism such as carbohydrate metabolism and lipid metabolism and down-regulation of pathways related to signal transduction for the heat-tolerant *B. calyciflorus* s.s. (Figure 5B, Figure S7). The opposite was observed for the heat-sensitive, *B fernandoi* , in which pathways related to signal transduction were up-regulated, while pathways related to , carbohydrate and lipid metabolism were down-regulation under high heat. Genes involved in meiosis pathway were also up-regulated under high heat, while genes involved in "ribosome" pathway were down-regulated for this species (Figure 5B, Figure S8).

3.6 Cross-species co-expression gene groups

To capture similar or contrasting patterns of expression between the two species, we searched for co-expression patterns in orthologous gene groups. Analysis with *Clust* revealed the presence of 8 gene groups/clusters (Figure S9). We have focused on just three of these because their patterns appear biologically relevant. Gene cluster C1 contained 150 orthogroups that had the same expression pattern between the two species and were up-regulated with increasing temperature (Figure 6). Five KEGG pathways were significantly enriched in the C1 group ($p<0.05$, FDR=0.05), including genes belonging to core metabolic pathways (KEGG pathway: 01100). Two clusters (C6 and C7) contained genes with opposing patterns of expression in the two species. In cluster C6, genes from the heat-tolerant *B.calyciflorus* s.s. had low expression at control conditions, were up-regulated at mild heat, and down-regulated again under high heat. In contrast, for*B. fernandoi* , genes in

C6 had higher expression under control conditions, were down-regulated at mild heat and up-regulated again at high heat. The longevity regulating pathway (KEGG pathway: 04212) was significantly enriched in C6 cluster ($p<0.05$, FDR=0.05). In cluster C7, for *B. calyciflorus* s.s. gene expression was high under control and mild heat conditions and down-regulated at high heat. However, for the heat-sensitive *B. fernandoi* , gene expression was low for control and mild heat conditions and up-regulated under high heat. In this cluster, a total number of 12 pathways were significantly enriched ($p<0.05$, FDR=0.05), among which pathways involved in signal transduction, nervous and endocrine systems, and replication and repair.

DISCUSSION

We combined life history and expression data to test variation in temperature-dependent responses between heat-tolerant and heat-sensitive species (Paraskevopoulou et al., 2018) from the recently resolved*B. calyciflorus* species complex (Papakostas et al., 2016). We observed a transcriptomic response that was largely consistent with phenotypic data from life history experiments. This has revealed both shared and species-specific patterns in gene expression in response to heat, and identified key functional pathways associated with temperate adaptation in these species. This demonstrates the power of testing the transcriptomic response of an organism to an environmental stressor by combining transcriptome with phenotypic data (DeBiasse & Kelly, 2016).

4.1 Life history heat response between heat-tolerant and heat-sensitive *Brachionus* species

Rotifers living in temporally variable habitats are exposed to frequent changes in their environment that may impact their life history. In both species we found a profound effect of temperature on survival; in both species increasing temperature led to reduced life span. Our results corroborate studies in other rotifer taxa that showed a profound effect of temperature on survival (Kauler & Enesco, 2011; Li et al., 2010; Ma, Xi, Zhang, Wen, & Xiang, 2010; Xiang, Jiang, Tao, Chen, & Xi, 2016; Xiang, Xi, Zhang, Ma, & Wen, 2010). Due to their ectothermic nature, rotifer body temperature increases with increasing ambient temperature, which accelerates metabolic rates. As a result, juvenile and egg development are accelerated up to a certain critical temperature limit, given unlimited food resources (Stelzer, 2017). Fecundity, on the other hand did not follow the same pattern. Fecundity was significantly reduced only at high heat exposure (*B. calyciflorus* s.s., 32°C; *B. fernandoi,* 26 °C), suggesting that fecundity is maintained across variable temperatures in both species, up to a limit above which conditions are too stressful.

Population growth rate (r) is considered a proxy to evaluate environmental specializations and stress response, representing the ability of rotifers to grow in a particular environment (Stelzer, 2005; Lowe, Kemp, Díaz-Avalos, & Montagnes, 2007; Kauler & Enesco, 2011; Weisse, Laufenstein, & Weithoff, 2013). Previous work in the rotifer complex of *Brachionus plicatilis* used population growth as a proxy to evaluate the salinity constrains between sibling species and found that - while species tolerated a wide range of salinities - their population growth rates respond differentially (Lowe et al. 2007; Gabaldón, Montero-Pau, Serra, & Carmona, 2013; Walczynska and Serra 2014). Based on this, it was suggested that growth rate variation implies specializations among species, which facilitate their dominance in different periods of the year and makes sympatric co-occurrence possible (Serra & Fontaneto, 2017). In rotifers, broad temperature tolerance has been found (Zhang et al., 2017; Li et al., 2010) which might reflect an adaptation to temperature fluctuations occurring in aquatic habitats. Both *B. calyciflorus* s.s. and *B. fernandoi* experience temperature fluctuations in their natural habitats and can survive a broad range of temperatures, however, their densities vary considerably relative to ambient temperature (Li et al., 2010; Wen et al., 2016; Zhang et al., 2017). High densities of *B. calyciflorus* s.s. have been reported during summer up to 32°C, while high densities of *B. fernandoi* have been reported during spring and winter even down to 4°C (Yang et al., 2017; Zhang et al., 2017). We have shown that although both species can survive a range of temperatures, both their population growth rate and expression of representative heat-stress genes is different. Our results further corroborates that these species are specialized in temperature tolerance, which might also translate into habitat specializations.

Differences in life histories between sibling species of the *B. calyciflorus* species complex have been found

in response to competition or/and predation risk (Wang et al., 2014). A recent study of Zhang, Lemmen, Zhou, Papakostas, and Declerck (2019) performed under stable food and temperature conditions (24 °C), showed differences in life history traits such as egg and juvenile developmental times, and egg production between these species. As a corollary, they demonstrated that observed differences are consistent across tested clones within species, i.e., they really represent differentiation between the species. According to our fitness results, our two species have evolved different strategies to respond to increased temperature, with *B. calyciflorus* adopting a life strategy of high population growth and low survival, as opposed to *B. fernandoi* with a strategy of low population growth and high survival. All above findings indicate that life histories of these two sibling species are differentially adapted to multiple stressors, biotic and abiotic, supporting the idea that the species are ecologically diverged and specialized for different environmental conditions, in particular with regard to temperature.

4.2 Heat shock response between heat-tolerant and heat-sensitive *Brachionus* species

Heat Shock Response, which involves the induction of heat shock protein (*hsp*) genes, is a well known and evolutionary conserved mechanism present in both prokaryotes and eukaryotes (Feder & Hofmann, 1999). Induction of *hsps* has been connected to several stress conditions such as exposure to extreme temperatures, heavy metals, pathogens, and osmotic stress (De Jong et al., 2008). In the present study, expression of *hsp* related genes mirrored measured changes in the population growth rate across a temperature gradient. Population growth rate was low when *hsp* genes were up-regulated. This pattern was consistent in both species, showing that *hsps* genes are indeed part of a species' stress response, when environmental conditions (here, temperature) are outside the 'comfort zone' for optimal growth.

Closely related species differentially adapted to cold *vs.* warm habitats have been found to express *hsp* genes differently also in other aquatic organisms such as the amphipods *Eulimnogammarus. S* pecies originated from a cold habitat when exposed to heat, up-regulated *hsp* genes already at lower temperatures compared to species from a warmer habitat (Bedulina et al. 2013). In the present study, closely related *Brachionus* species with differences in their ability to tolerate heat have been found to express *hsp*genes differently. More specifically, hsp genes were induced outside the temperature of optimal growth: in the heat-sensitive *B. fernandoi, hsp* genes were induced by heat, while in heat-tolerant *B. calyciflorus* s.s. the majority of *hsp* genes were induced at the lower end of temperature exposure (20 °C), indicating that 20 °C may already be cold stress for the heat-tolerant species.

Proteins of the *hsp90* family serve to increase the available chaperons in the cells in order to recover from cellular stress and maintain structural integrity at high temperatures. In contrast to other hsp genes, their expression patterns supported a specific involvement in heat response, as genes encoding for *hsp90* were up-regulated towards the higher temperature regime in both species. The expression of*hsp90* gene has been found to be temperature dependent also in other aquatic organisms, e.g. copepods, oysters (Schoville et al., 2012; Kim et al., 2017; Lim et al., 2016). This suggests that induction of*hsp90* gene along with heat might be a common mechanism in aquatic organisms. In *B. calyciflorus* s.s., genes encoding *hsp20*were also induced by high heat. Up-regulation of *hsp20* genes has been reported previously from other *Brachionus* species and copepods as a response to elevated temperatures (Rhee et al., 2011; Seo, Lee, Park, & Lee, 2006). Transformed bacteria (*Escherichia coli*) expressing the *Brachionus hsp20* (*Br-hsps20*) gene had a 100 times increase in their survival compared to the non transformed ones under high heat-stress, indicating that *Br-hsp20*specifically contributes to increased thermal tolerance (Rhee et al., 2011). Up-regulation of *hsp20* genes was *found to* also increase resistance to oxidative stress (Rhee et al. 2011). Possibly, an increase in expression of *hsp20* reflects a cellular defense mechanism in response to different stressors that might be common among*Brachionus* species.

A reversed expression pattern among our two species was found in genes encoding for *hsp70* and *hsp27* . In heat-tolerant *B. calyciflorus* s.s., genes encoding for *hsp70* were significantly up-regulated towards the lower temperature, while in *B. fernandoi*towards the higher temperature. It seems that temperatures such as 20°C might constitute cold stress for warm adapted species and a stress response might be initiated under these conditions. In *B. calyciflorus* s.s., the same induction pattern with*hsp70* genes followed genes encoding for

hsp40 and*hsp60* pointing towards a synergistic mechanism regulating the expression of these three genes as reported also in *B. manjavacas*(Smith et al., 2012). Such a synergistic relationship has been reported among *hsp40* and *hsp70* proteins, as *hsp40* regulates the ATPase activity of *hsp70* (Cintron & Toft, 2006). These genes were induced in *B. manjavacas* with increasing heat (Smith et al., 2012). This indicates that these genes are induced under any condition that constitutes temperature stress for a particular *Brachionus*species.

4.3 Metabolic temperature response between heat-tolerant and heat-sensitive *Brachionus* species

Metabolism in ectotherms is inextricably linked to environmental temperature and its rate is accelerated by temperature increase. Genes belonging to core metabolic pathways had, in general, the same expression pattern between the heat-tolerant *B. calyciflorus* s.s. and the heat sensitive *B. fernandoi,* showing an increased expression with temperature induction. However, there were significant differences between the species in genes involved in oxidative phosphorylation, lipid metabolism, and carbohydrate metabolism.

Oxidative stress is related to the production of toxic compounds that are called reactive oxygen species (ROS) which cause cellular response by modifying proteins and nucleic acids and contributes to cellular damage (Halliwell & Whiteman, 2004). Exposure of ectothermic organisms to elevated temperatures accelerates mitochondrial respiration and potentially increases ROS formation (Heise, Puntarulo, Pörtner, & Abele, 2003; Keller, Sommer, Pörtner, & Abele, 2004). ROS formation blocks heat shock response and refolding activity under heat stress, thereby leading to increasing cellular stress and ultimately heat sensitivity (Adachi et al., 2009). Genes related to oxidative stress were significantly up-regulated over temperature increase in heat-sensitive *B. fernandoi.* In contrast, these genes were significantly down-regulated over temperature increase in the heat-tolerant *B. calysiflorus* s.s.. Here, oxidative stress response was induced at the lowest temperature tested here (20°C). Among the differentially expressed genes were NADH dehydrogenase and glutathione S-transferase (GST). For the *B. calyciflorus* s.s., a significant induction (7x fold change) of NADH dehydrogonase has been reported after sustained cold stress on 14 °C for 30 days (Paraskevopoulou, Dennis, Weithoff, Hartmann, & Tiedemann, 2019). Transcriptional regulation of GST genes also differed in two copepod species of the genus *Tigriopus* . For the species *T. japonicas,* GST genes were significantly down-regulated in response to temperature elevation up to 35 °C (Han, Jeong, Byeon, & Lee, 2018), In Pacific oyster, both of the above genes were down-regulated, with diversity in their expression according to the duration of heat exposure. This indicates that repression of oxidation stress likely acts as a protective mechanism of the cells and potentially enhances heat tolerance (Lim et al, 2016).

Lipid and carbohydrate metabolism are highly conserved processes that affect nearly all aspects of an organism's biology. The consumed lipids and carbohydrates are broken down during digestion into fatty acids and simple sugars, providing the essentials to produce a wide range of metabolites that are required for development and survival. Genes related to lipid and carbohydrate metabolism were up-regulated from mild to high heat in heat-tolerant, *B. calyciflorus* s.s. and down-regulated in heat-sensitive *B. fernandoi* . Up-regulation with heat of genes related to carbohydrate metabolism was also found in a teleost fish, *Gillichthys mirabilis* (Buckley, Gracey, & Somero, 2007) and in the Pacific oyster, *Crassostrea gigas* , after 6h of acute heat exposure (Yang, Gao, Liu, Wang, & Zhou, 2017). This suggests a need for rapid production of ATP under increasing temperatures. Apparently, *B. calyciflorus* s.s. has adapted to maintain its metabolism under high heat, while the heat-sensitive species *B. fernandoi* apparently shuts down costly metabolic processes (indicated by down-regulation of the majority of metabolic related pathways) in order to allocate available resources to survival.

4.4 Ribosomal response between heat-tolerant and heat-sensitive*Brachionus* species

Ribosome biogenesis is a complex and energy-demanding process requiring coordination of ribosomal RNA (rRNA) and ribosomal protein production. Ribosomal proteins have been mentioned several times to participate in stress response and they have been either induced or suppressed upon temperature increase (Lim et al., 2016; Podrabsky & Somero, 2004; Schoville et al., 2012; Truebano et al., 2010). Ribosomal protein related genes, in heat tolerant *B. calyciflorus* s.s., were up-regulated towards the lower imposed temperature here

at 20°C, indicating again that 20 °C likely comprise stressful conditions for this species. In contrast, in the heat-sensitive *B. fernandoi* , ribosomal protein related genes were up-regulated under mild heat stress (23 °C), suggesting an increased translation capacity or a protection of ribosomal function through the addition or replacement of ribosomal proteins (Meistertzheim, Tanguy, Moraga, & Thébault, 2007). However, further temperature increase up to 26 °C, resulted in down-regulation of ribosomal related genes. This suppression of protein biosynthesis may reflect cellular homeostasis or an energy saving mechanism to cope with thermal stress, as protein metabolism consumes a large amount of ATP.

4.5 Other molecular mechanisms of heat response in heat-tolerant and heat-sensitive *Brachionus* species

Brachionus , as most monogonont rotifers, have two reproductive modes, one asexual allowing for fast population growth and one sexual to promote recombination under unfavorable environmental conditions (Gilbert, 1974; Schröder, 2005). The sexual phase of reproduction is generally induced due to environmental factors such as photoperiod, population density and food composition (Gilbert, 1974; Pourriot & Snell, 1983; Schröder, 2005). Rotifer species are capable of abandoning either the sexual or the asexual phase. Abandoning asexuality is very rare, however, abandoning sexuality is a common phenomenon in clones that have been under laboratory cultivation over a long period of time and it reliess on a recessive allele (Stelzer, 2008; Stelzer, Schmidt, Wiedlroither, & Riss, 2010). In *B. fernandoi* , increase of temperature resulted in significant up-regulation of genes related to meiosis, indicating that temperature exposure above 23 °C triggered sexual reproduction. In *B. calyciflorus* s.s., there was no significant up-regulation of meiosis-related genes, neither at high nor at low temperatures. Possible explanations are that this clone has lost the ability of sexual reproduction or that sexual reproduction is triggered by temperatures beyond the range tested here or stimuli other than temperature.

Epigenetic control on transcription can be achieved by many mechanisms, including DNA methylation or post-translational modifications to histone tails, including histone methylation and acetylation. It is known from genomic/transcriptomic studies on *B. manjavacas* and other rotifers that rotifers lack DNA methyltransferases (Dnmt1, Dnmt3) for epigenetic transcriptional regulation (Gribble, Mark Welch, 2017; Kim et al., 2016). However, they do not lack the molecular machinery for post-translational regulation to histone tails, which play an important role in regulating gene expression. In *B . fernandoi* , we found that exposure to high temperatures resulted in up-regulation of histone related methyltransferase genes (H3K4, H3K79, H4K20). Trimethylation of H3K4 and H3K79 are associated with activation of transcription, while methylation of H4K20 has been related to silencing chromatin (Hyun, Jeon, Park, & Kim, 2017). Silencing of chromatin might be related to translation suppression that we found for the same species under the same conditions of heat exposure. Transcriptional activation via trimethylation of H3K79 and H3K4 might be associated with a numerous environmental information processing pathways that were up-regulated in this species under high heat exposure.

CONCLUSIONS

In conclusion, we found significantly different responses to heat between heat-tolerant and heat-sensitive *Brachionus* species. Transcriptomic responses were found to correlate with differences in fitness and especially differences in population growth, indicating underlying mechanisms of phenotypes' responses to environmental change. Generally, the respective species upregulated metabolism/translation related genes under the temperature with highest growth rate, while stress related (and – in one species – meiosis related) genes were expressed beyond the temperature regime optimal for growth. What had been historically considered the single species *B. calyciflorus*actually comprises several closely related rotifer species, which are differentially adapted to different environmental conditions (here, temperature) regarding their gene expression profiles and can hence occur in sympatry. The genes found to be upregulated under heat stress might be targets of selection potentially contributing to the ecological divergence of the two species. Additionally, their expression profiles might be used as biomarkers to assess species vulnerability to environmental conditions and climate changes.

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DATA ACCESSIBILITY

All SRA files are available from the Gen Bank database (accession numbers: SRR10426055-76). All other data that support the findings of this study will be deposited on Dryad repository.

AUTHOR CONTRIBUTIONS

The project was conceived by R.T. S.P, and G.W. The lab work and the life-table experiments were performed by S.P. Data analysis and interpretation of results was carried out by S.P. and A.B.D, with input from R.T.. The manuscript was drafted by S.P. Further editing and manuscript finalization was coordinated by S.P, with contributions from all authors. All contributing authors read and agreed to the final version of the manuscript.

TABLE 1: Summary statistics of the *de novo*assemblies of the *Brachionus calyciflorus* s.s. and *B. fernandoi* transcriptomes.

Transcriptome assembly statistics	*B. calyciflorus* **s.s.**	B. fernandoi
# Raw reads (n)	798,693,466	430,046,346
# Trimmed and high quality raw reads assembled (n)	670,242,336	358,527,278
# assembled contigs (n)	144,037	224,735
# assembled contigs after contamination removal (n)	128,999	187.245
# assembled “unigenes” (n)	72,165	94,884
# predicted ORFs (n)	17,973	19,440
Average length (bp)	1013.05	854.87
Median length (bp)	526	457
Total assembled bases (bp)	130,682,421	160,070,295
N50 (bp)	1,827	1,490
GC content for the entire assembly (%)	28.19	30.31
# KO terms	5,947	6,477

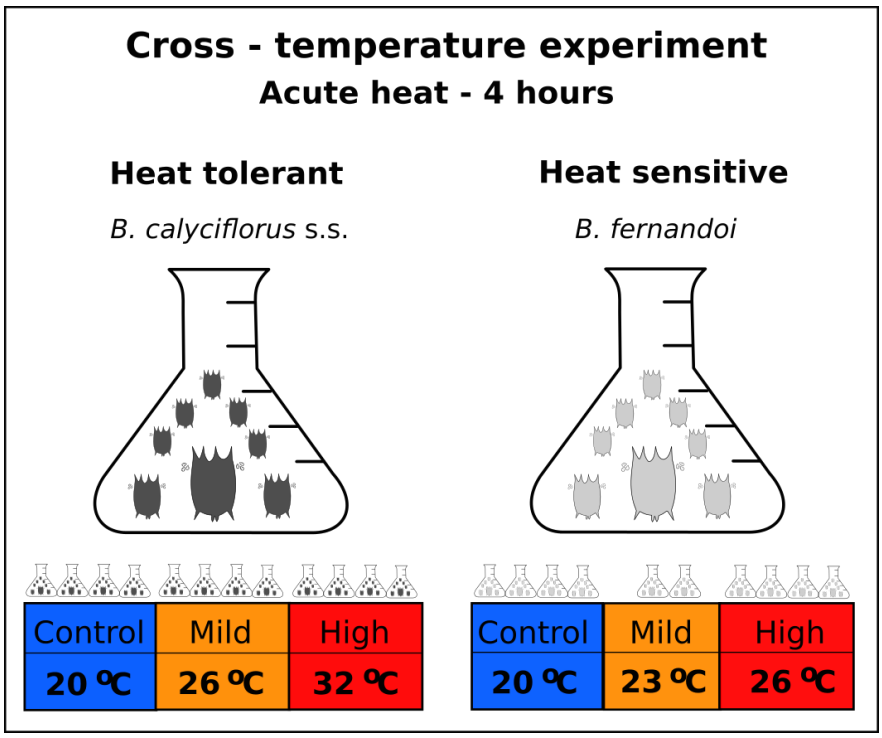


Figure 1. Experimental design for transcriptomic responses to heat exposure between heat-tolerant and the heat-sensitive*Brachionous* species. Temperature regimes were chosen to represent control (20 °C), mild (26/23 °C) and high (32/26 °C) heat for each species.

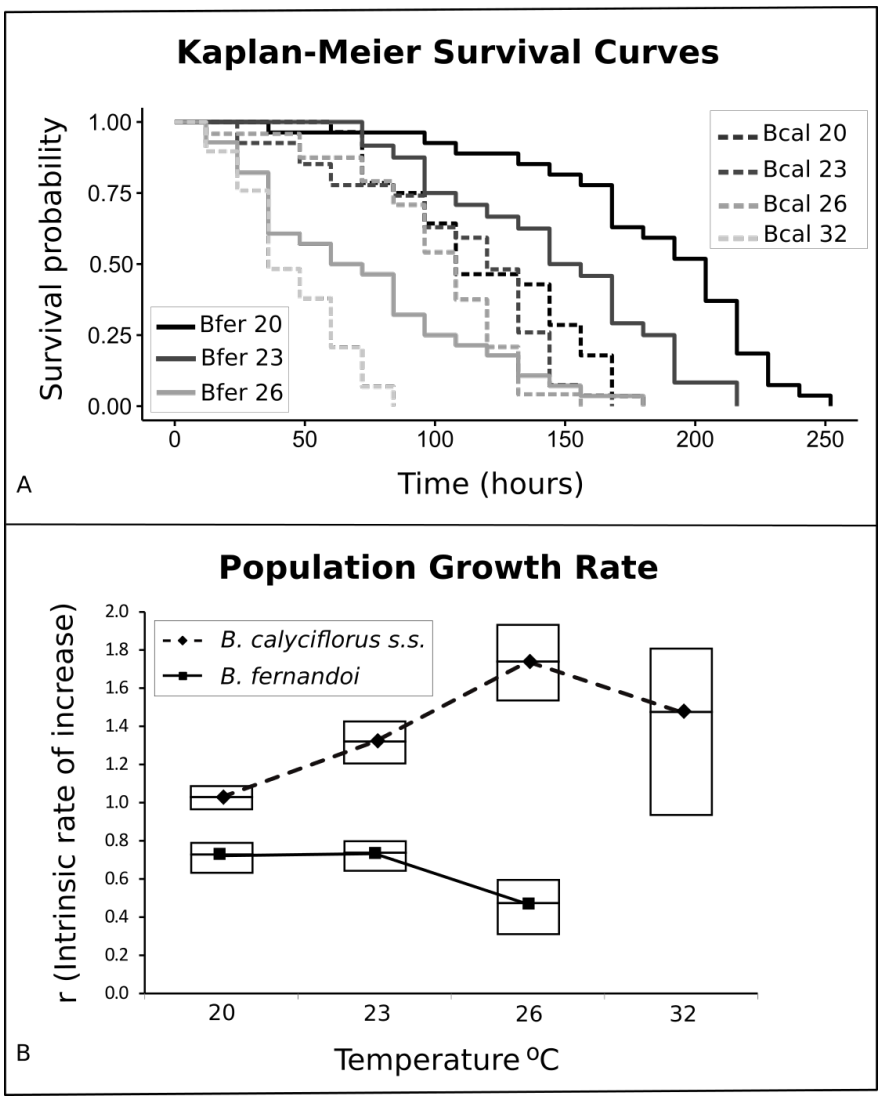
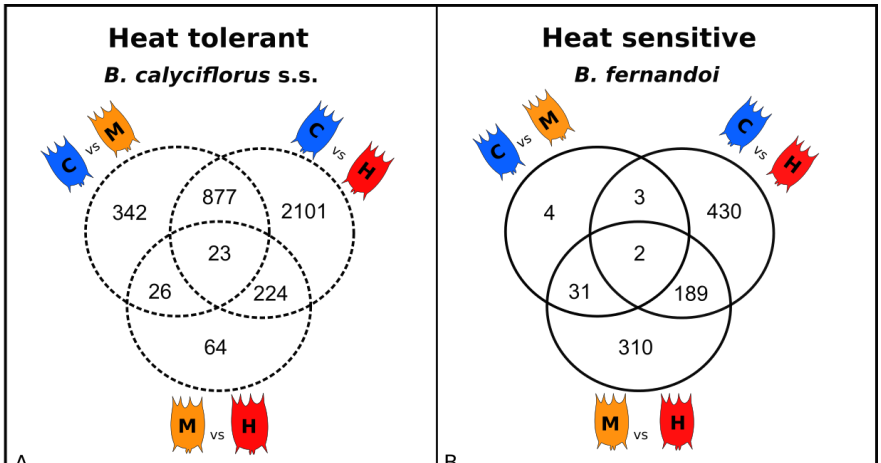


Figure 2. Kaplan-Meier survival curves (A), and Population growth rate/r (B) for the heat-tolerant *B. calyciflorus*s.s. (Bcal) and the heat-sensitive *B. fernandoi* (Bfer) under different temperature conditions. Solid lines denote responses of*B. fernandoi* to different temperatures (20, 23, 26), while dashed lines represent responses of *B. calyciflorus* s.s (20, 23, 26, 32). Boxes denote the 95% confidence intervals estimated via a bootstrap procedure with 199 iterations.



	total	up regulation	down regulation		total	up regulation	down regulation
C vs M	1268	476	792	C vs M	40	6	34
C vs H	3225	1529	1696	C vs H	624	227	397
M vs H	337	177	160	M vs H	532	373	159

Figure 3. Venn diagrams showing the number of differentially expressed genes in pairwise temperature comparisons as determine by the DESeq2 analyses (FDR=0.05) for the heat-tolerant *B. calyciflorus*s.s. (A) and the heat-sensitive *B. fernandoi* (B). Blue color (C) represents control treatment, orange color (M) represents mild heat treatment, and red color (H) represents high heat treatment. Up- and down- regulation is defined from the perspective of the higher temperature over the lower one.

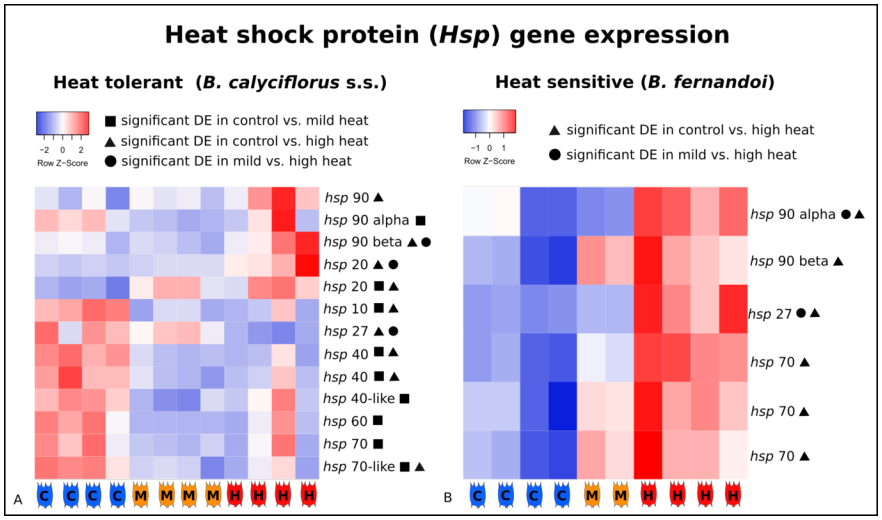


Figure 4. Heat map using normalized counts which show patterns of expression in *hsp* genes for the heat-tolerant *B. calyciflorus* s.s. (A), and the heat-sensitive *B. fernandoi* (B). Blue color (C) represents control treatment, orange color (M) represents mild heat treatment, and red color (H) represents high heat treatment. The symbols indicate the pairwise comparison in which differential gene expression (DE) was

significant ($p<0.05$). The color key represents a spectrum of lowest gene expression (blue) to highest gene expression (red).

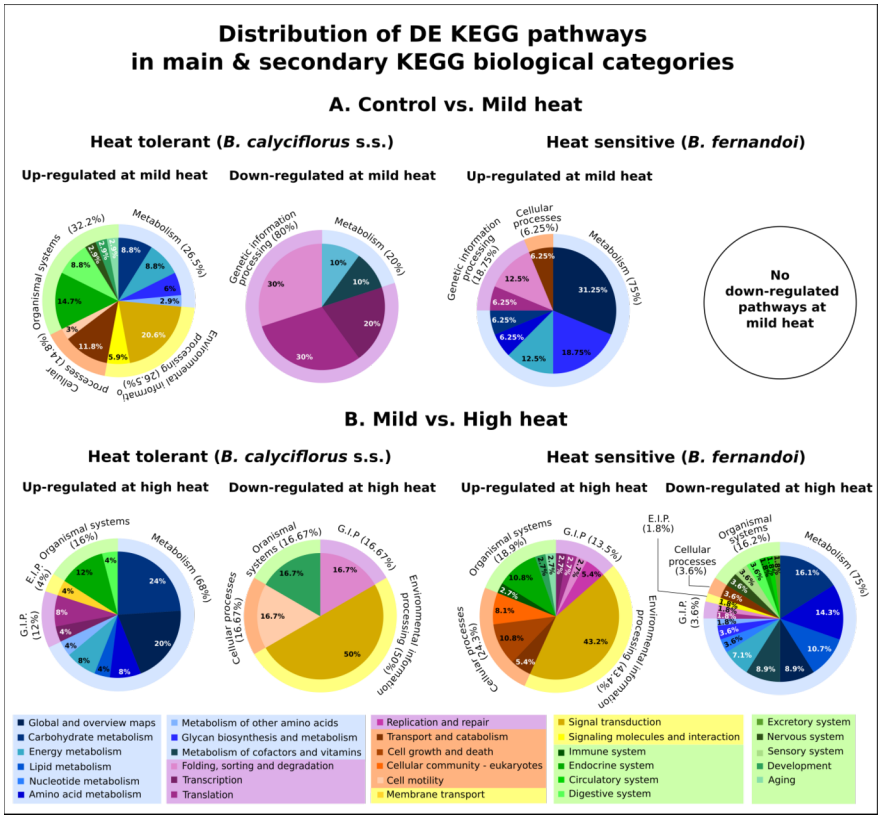


Figure 5. Distribution of differential expressed (DE) KEGG pathways (FDR=0.05) in main and secondary KEGG biological categories in control *vs.* mild heat exposure (A), and in mild *vs.* high heat exposure (B). Left side represent changes captured for the heat tolerant, *B. calyciflorus* s.s. while right side for the heat-sensitive *B. fernandoi* .

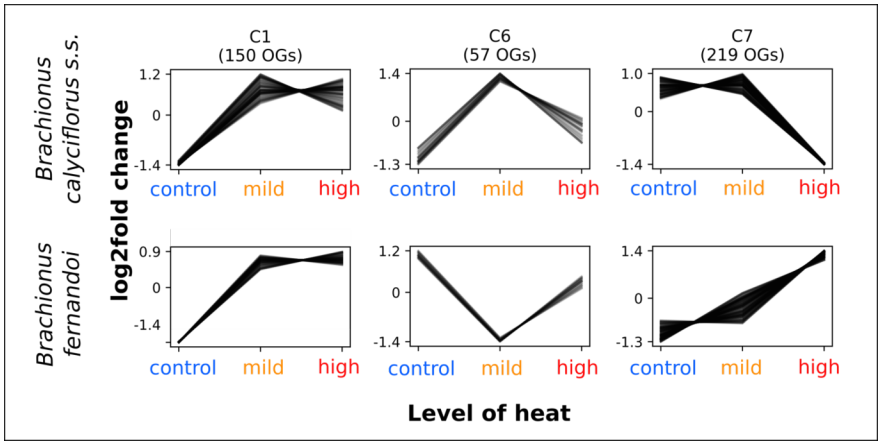


Figure 6. Shared and contrasting co-expression patterns of gene orthogroups (OGs) between heat-tolerant

and heat-sensitive*Brachionus* species. C1, C6, C7 represent the numbers of clusters produced by *Clust* program. The total number and co-expression pattern of all clusters are given in Figure S9.