

# Novel microRNAs are associated with population divergence in transcriptional response to thermal stress in an intertidal copepod

Allie M. Graham  | Felipe S. Barreto 

Department of Integrative Biology, Oregon State University, Corvallis, Oregon

## Correspondence

Allie M. Graham, Department of Integrative Biology, Oregon State University, Corvallis, OR.

Emails: graham.allie@gmail.com; grahaall@oregonstate.edu

## Funding information

National Science Foundation, Grant/Award Number: DEB-1556455

## Abstract

The role of gene expression in adaptation to differing thermal environments has been assayed extensively. Yet, in most natural systems, analyses of gene expression reveal only one level of the complexity of regulatory machineries. MicroRNAs (miRNAs) are small noncoding RNAs which are key components of many gene regulatory networks, and they play important roles in a variety of cellular pathways by modulating post-transcriptional quantities of mRNA available for protein synthesis. The characterization of miRNA loci and their regulatory dynamics in nonmodel systems are still largely understudied. In this study, we examine the role of miRNAs in response to high thermal stress in the intertidal copepod *Tigriopus californicus*. Allopatric populations of this species show varying levels of local adaptation with respect to thermal regimes, and previous studies showed divergence in gene expression between populations from very different thermal environments. We examined the transcriptional response to temperature stress in two populations separated by only 8 km by utilizing RNA-seq to quantify both mRNA and miRNA levels. Using the currently available genome sequence, we first describe the repertoire of miRNAs in *T. californicus* and assess the degree to which transcriptional response to temperature stress is governed by miRNA activity. The two populations showed large differences in the number of genes involved, the magnitude of change in commonly used genes and in the number of miRNAs involved in transcriptional modulation during stress. Our results suggest that an increased level of regulatory network complexity may underlie improved survivorship under thermal stress in one of the populations.

## KEYWORDS

gene regulation, microRNA, temperature stress, *Tigriopus californicus*

## 1 | INTRODUCTION

Environmental heterogeneity along the geographic range of a species can set the stage for natural selection to vary in strength and direction among allopatric populations, frequently resulting in local adaptation (Hedrick, 2006; Nosil, Funk, & Ortiz-Barrientos, 2009). Variation in temperature regimes is of obvious importance in any

biological system because of the strong dependence of rates of cellular function on optimal temperature ranges, and the immediate damaging effects of elevated temperature on protein conformation and function. Adaptation to thermal environment has been widely examined at the structural level, in which amino acid substitutions can have a direct impact on cellular function under different temperature conditions (Keller & Seehausen, 2012; Kingsolver, 2009).

However, selection on regulatory variation is known to play a major role in adaptation (Carroll, 2008; Levine & Tjian, 2003; Wittkopp & Kalay, 2011), likely because changes in gene expression require simple changes in few regulatory elements, thus producing widespread downstream effects along a functional pathway (Fraser, 2013; Kenkel & Matz, 2017; Whitehead, Triant, Champlin, & Nacci, 2010). Investigations of genomewide transcriptional profiling have become easily accessible to nonmodel systems via RNA-seq, and current efforts focus on describing additional levels of regulation in order to understand the complexity of gene interaction networks (Cork & Purugganan, 2004).

MicroRNAs (miRNAs) are small noncoding RNAs that are ~23 nt long and are key components of gene regulatory networks (Bartel, 2009; Berezikov, 2011). Although the dynamics and evolution of miRNAs are still being elucidated, they have been implicated in the regulation of virtually every biological process found in multicellular eukaryotes (Bartel, 2009). Typically, mature miRNAs negatively regulate the translation of protein-coding genes by imperfect binding to complementary sites in the 3' untranslated regions (UTRs) of messenger RNAs, subjecting the transcript to either cleavage or to blockage of its translation. MicroRNAs are well recognized for their role in development (Emde & Hornstein, 2014; Leung & Sharp, 2010), but multiple studies have suggested they play important roles in a variety of stress responses, including nutrient deprivation (Sunkar, Chinnusamy, Zhu, & Zhu, 2007), hibernation/torpor (Luu, Biggar, Wu, & Storey, 2016), DNA damage (Hu & Gatti, 2010), oxidative stress/hypoxia (Kulshreshtha et al., 2007), salinity (Gao et al., 2011) and temperature (Gajigan & Conaco, 2017; Nehammer, Podolska, Mackowiak, Kagias, & Pockock, 2015). In addition, miRNAs have the potential to facilitate evolutionary change via changes in expression of miRNA target genes (Li & Zhang, 2013; Peterson, Dietrich, & McPeck, 2009) and thus may be co-opted in mechanisms associated with adaptation and population divergence (Arif et al., 2013; Franchini, Xiong, Fruciano, & Meyer, 2016; Jovelin & Cutter, 2014). The role of miRNAs in population divergence is still largely unstudied in natural systems; thus, miRNA repertoire and their respective activity need to be addressed at the population level.

The copepod *Tigriopus californicus* is an ideal organism in which to investigate both local adaptation and population divergence. It is an abundant resident of high intertidal rock pools ranging from Alaska to Baja California, Mexico, and for which genetic analyses have demonstrated highly restricted gene flow among populations (Burton, 1997; Burton & Lee, 1994; Edmands, 2001). Genetic divergence is particularly pronounced in mtDNA, with populations differing by as much as ~25% (Burton, Byrne, & Rawson, 2007). Because the pools they inhabit are very shallow and only infrequently refreshed by high tides, these copepods are under considerable environmental stress, in the form of temperature, salinity, pH and oxygen fluctuations. Recent experiments in this system have shown that, while tolerance to acute temperature stress follows a latitudinal cline coarsely (Tangwancharoen & Burton, 2014; Willett, 2010), analyses at finer geographic scales reveal a mosaic pattern of temperature tolerance (Kelly, Sanford, & Grosberg, 2012; Pereira,

Barreto, & Burton, 2014), suggesting that microhabitat differences play an important role in adaptive divergence among populations. Genomic analyses examining responses to thermal stress have focused on gene expression differences between highly divergent and geographically distant populations (Kelly, Pankey, DeBiasse, & Plachetzki, 2017; Schoville, Barreto, Moy, Wolff, & Burton, 2012). These studies found widespread differentiation in transcription between heat-tolerant Southern California populations and more sensitive Northern California populations. It remains unclear, however, how much divergence exists at smaller scales and to what extent geographically close populations share stress response mechanisms.

In a study comparing hybrids to their parental stocks, Pereira et al. (2014) found striking levels of transgressive segregation for high thermotolerance (i.e., some hybrids survive at significantly higher rates than their parents) in a cross between two Southern California populations (San Diego and Bird Rock) that are separated by only ~8 km of coastline and are both highly thermotolerant. The predicted genomic architecture for transgressive segregation involves complementary action of different genes in a novel hybrid genome (Rieseberg, Archer, & Wayne, 1999). Therefore, we hypothesize that these two neighbouring yet isolated populations have evolved high thermotolerance through different genetic mechanisms and likely show divergence at the regulatory level.

In this study, we test the transcriptional response to temperature stress of these two southern populations of *T. californicus*, by utilizing RNA-seq paired with targeted miRNA sequencing. Here, we (a) characterize miRNA diversity within the genome of *T. californicus*, (b) identify differentially expressed mRNAs and miRNAs and (c) determine the extent to which miRNAs are associated with the transcriptional response to temperature stress across populations. Ultimately, we characterize 37 conserved and 111 novel miRNAs across the *T. californicus* genome, and we find that few novel miRNAs show divergence regulation between the two populations in response to high temperature.

## 2 | MATERIALS AND METHODS

### 2.1 | Copepod collection and thermal stress experiments

Copepods were collected from high intertidal rocky pools in San Diego, California: Ocean Beach (SD: 32°44'N, 117°15'W), and Bird Rock (BR: 32°48'N, 117°16'W). Cultures were maintained at common garden conditions in incubators (20°C, 12 hr: 12 hr light: dark cycle) and in multiple 400-ml beakers with artificial sea water (ASW; Instant Ocean) at 35 parts-per-thousand salinity. Copepods were fed a mixture of the live microalga *Isochrysis galbana* and *Tetraselmis suecica* and finely ground dry fish flakes (Tetramin). Once per month, water and food were changed. Populations were maintained at approximately 400–600 individuals per beaker, and beakers from each population were periodically remixed to maintain outbreeding. We also obtained field temperature profiles by installing Thermochron iButton data loggers (Maxim Integrated Products) in pools inhabited by *T. californicus* in both of these

sites. Data loggers were affixed to the bottom of shallow *T. californicus* pools using marine epoxy (Pettit Splash Zone) and were programmed to collect data at 30-min intervals for 25 consecutive days in March/April and then in July 2015. Concordant with previous reports (Kelly et al., 2012; Leong, Sun, & Edmands, 2017), temperature in these shallow pools frequently reached >30°C even in March, with a maximum of 38°C in July (Supporting information Figure S1).

In order to remove effects from recent environmental experiences and to quantify only heritable variation, we maintained copepods in laboratory cultures for at least three generations before performing any experiment. To test the reproducibility of the levels of thermotolerance of these two populations described by Pereira et al. (2014) and Willett (2010), we assessed their survivorship after acute stress across a range of temperatures. Twenty adult copepods (equal sex ratios) from the acclimated cultures were placed in 5 ml ASW in 15-ml Falcon tubes and immersed in a water bath at a target temperature for 1 hr. After the heat stress, tubes were immersed in 20°C water for another hour and then copepods were transferred to culture plates with food. Target temperatures ranged from 34°C to 40°C, and mortality was assayed after three days. Number of replicates ranged from 4 to 6 for the high- and low-end temperatures, where survivorship tends to be consistent. The number was increased to 10–14 for the intermediate temperatures, since more survivorship variation occurs in these temperatures and hence additional replicates should increase accuracy. The fraction of individuals alive or dead in each replicate were regressed on temperature using a probit function to estimate LD<sub>50</sub> for each population.

For transcriptomic analyses, approximately 250 adult copepods were taken from each population stock and allowed to acclimate in separate beakers for 2 days. On the day of the experiment, the copepods were then distributed across six experimental replicates per population, with 30–35 individuals per replicate at approximately equal sex ratios. Copepods were placed in 5 ml filtered ASW in 15-ml Falcon tubes, which were then immersed in water at 20°C (control, 3 replicates) or 33°C (heat stress, 3 replicates) for 1 hr. This temperature was selected because it is near the high thermal limit, and hence stressful, but it is still sublethal (Pereira et al., 2014 and this study), which is important in order to avoid quantifying gene expression in nearly dead copepods.

## 2.2 | RNA extraction and sequencing

Total RNA from each of the 12 samples was extracted by first homogenizing tissue in TRIzol® with 1 mm zirconia-silica beads (Biospec) and then purified with Direct-zol RNA MiniPrep Kit (Zymo Research), following the manufacturer's protocol. RNA was DNase-treated with TURBO DNase (Thermo Fisher) and then cleaned up with RNA Clean and Concentrator Kit (Zymo Research). The RNA from each replicate was then split in two aliquots of 300–500 ng, one to be used for small RNA-specific sequencing and the other for traditional mRNA-seq. For miRNAs, the libraries were created using NEBNext® Small RNA Library Prep Set for Illumina®, while mRNA libraries were prepared using the NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina®. Each set of libraries (12 small

RNA and 12 mRNA) was pooled and each sequenced on one lane of the Illumina HiSeq3000 platform as 50-bp single-end reads (Oregon State University Center for Genome Research and Biocomputing), for a total of 2 lanes.

## 2.3 | miRNA identification and expression

The small RNA sequencing data were trimmed of adapter sequences, then filtered by length and quality using cutadapt (Martin, 2011), such that reads of length greater than or equal to 15 nt with an average Phred score of 30 were retained for further analysis. miRDeep2 was used to identify sequences as potential microRNAs, functioning as a pipeline for automating a number of steps (Friedländer, Mackowiak, Li, Chen, & Rajewsky, 2011; Mackowiak, 2011). The trimmed reads were mapped to the genome of *T. californicus* (Barreto et al., 2018) using bowtie (Langmead & Salzberg, 2012). Potential pre-miRNA precursor sequences were then identified and folded using RNAfold, which determines presence of a hairpin secondary structure (Lorenz et al., 2011), as well as whether the precursor is energetically stable using randfold (Bonnet, Wuyts, Rouzé, & Peer, 2004). Predicted pre-miRNAs that had a miRDeep2 score above 10, in addition to having mature/star sequences between 20 and 26 nt in length, and having a significant randfold *P*-value, were retained for further analyses. The *p*-value associated with randfold is based on whether potential precursors (a) fold into an un-bifurcated hairpin, (b) can be partitioned into candidate mature, loop and star part based on the reads mapping to it and (c) have minimum 60% of the nucleotides in the candidate mature sequence be base-paired when in hairpin formation. In addition, miRNA annotation criteria were applied (Fromm et al., 2015), specifically, the expression of 20–26 nucleotide long reads for both strands, a minimum of 16-nt complementarity between the two arms, at least 8 nt in the loop sequence, and the presence of a 2-nt 3' overhang for both mature and star strands. A BLASTN search was performed on putative miRNAs against all mature miRNAs available through miRBase (database downloaded January 2018) in order to identify miRNA homologs. This was performed on 4 different samples (one from each population and treatment), which had the largest number of reads (BR33-1, BRcon-3, SD33-3, SDcon-2; Supporting information Table S1), in order to maximize our ability to detect most miRNAs. Ultimately, a master list of putative miRNAs in the *T. californicus* genome was created by consolidating the lists from all 4 samples. This list comprises miRNAs which (a) met all requirements, that is, "bona-fide," (b) met all but one requirement and had BLASTN results to a known miRNA, that is, "retained," and (c) met all but one requirement and did not have a BLASTN result to a known miRNA, that is, "equivocal."

To quantify the expression level of each annotated miRNA, trimmed reads from each small RNA library were mapped to the *T. californicus* genome, and the number of reads mapping to predicted miRNA precursors was counted using the quantifier.pl script in miRDeep2. Differentially expressed miRNAs in control (20°C) versus elevated temperature (33°C) were determined using edgeR (Robinson, McCarthy, & Smyth, 2010), after normalization with

trimmed mean of M-values (TMM; Robinson & Oshlack, 2010). Normalized miRNA expression levels were also used in a principal component analysis (PCA) to assess the overall variation among replicates and treatments in each population (Supporting information Figure S2). Ultimately, miRNAs with an FDR-adjusted  $p$ -value  $\leq 0.05$  were considered to be differentially expressed.

The locations of the miRNAs were determined by cross-referencing the *T. californicus* genome with the coordinates of all miRNAs, using bedtools (Quinlan & Hall, 2010); miRNA locations that were characterized as intergenic were those that did not intersect with any annotated gene in the genome, while intronic locations were those that intersected with a gene but were not annotated as either an exon or an UTR.

Specific commands and scripts utilized in these analyses are available at <https://github.com/amgraham07>.

## 2.4 | Determination of miRNA targets and Gene Ontology enrichment

Targets of the miRNAs were then predicted using MIRANDA (Enright et al., 2003), which determines putative targets using two criteria: (a) the complementarity of mature miRNA to a given mRNA and (b) estimations of free energy formation ( $\Delta G_{\text{duplex}}$ ) of the miRNA:mRNA duplex. Due to the fact that miRNAs function via binding to the 3' UTR of target mRNAs, we used as input the 3' UTR sequences predicted by the annotation of *T. californicus* transcripts. A total of 10,296 genes had 3'UTR annotations that were  $\geq 25$  nt, and these were recovered from the genome sequence for use in miRanda. In identifying potential miRNA targets, we utilized a conservative approach, which involved reporting matches that met the following criteria: (a) strict seed binding of  $\Delta G_{\text{duplex}} \leq -10$  kcal/mole, and (b) only targets with an exact seed match and an A in position 1 (Gajigan & Conaco, 2017).

Gene Ontology (GO) annotation and enrichment of sequence matches were retrieved using Blast2GO (Conesa et al., 2005) with a threshold  $p$ -value  $< 0.05$ . Overrepresented GO terms were assayed in two different ways. First, the list of all putative targets identified by miRanda were compared against the rest of the genes in the *T. californicus* genome. Second, the subset of targets that were significantly differentially expressed were compared against the rest of the genes in the genome.

## 2.5 | Transcriptome sequencing and differential gene expression analysis

The mRNA reads were trimmed of adapter sequences, then filtered by length and quality using cutadapt (Martin, 2011), such that reads of length greater than or equal to 30 nt with an average Phred score of 30 were retained for further analysis. We used Salmon aligner (Patro, Duggal, Love, Irizarry, & Kingsford, 2017) to map reads from each library to reference transcriptomes. Since this species is known for high levels of sequence divergence among populations, use of a single reference transcriptome is not ideal for mapping of reads from different populations. Barreto et al. (2018) assembled transcriptomes

specific to each of these populations using the species reference genome, an approach that also results in good orthology correspondence between population sequences. We thus used these SD- and BR-specific transcriptomes as references for mapping reads from their respective populations. As with miRNA, tests for differential expression of mRNAs between control (20°C) and elevated temperature (33°C) for each population were determined using edgeR with TMM normalization, and PCA used to assess overall distribution of replicates (Supporting information Figure S2). Only genes with a minimum of three counts per million mapped reads in at least three libraries were included in the analysis. Transcripts with an FDR-adjusted  $p$ -value  $\leq 0.05$  were considered to be differentially expressed.

Enrichment of GO terms was assessed with Blast2GO with a threshold  $p$ -value  $\leq 0.05$ , by comparing (a) the set of significantly up-regulated genes and (b) the set of significantly downregulated genes against the remainder of the genes.

After identifying putative targets based on binding predictions above, we took advantage of our matched small RNA and mRNA sequencing libraries to further examine miRNA-mRNA relationships. We used the package MIRLAB (Le, Zhang, Liu, Liu, & Li, 2015) to test for Pearson correlations in expression levels between differentially expressed miRNA and their differentially expressed predicted target mRNAs.

## 2.6 | Quantitative PCR of miRNA

We used quantitative reverse transcription PCR (RT-qPCR) to attempt to validate expression change in the four novel miRNA detected to respond to heat stress (see Results). Since mature miRNAs identified here are only 22 nucleotides in length, sequence similarity between different loci may complicate PCR primer design. We first assessed sequence similarity among each of the four miRNA and all other miRNAs identified by performing pairwise alignments. Following the methods of Shi and Chiang (2005) and Balcells, Cirera, and Busk (2011), primers were designed manually and incorporated single nucleotide differences when needed.

For RT-qPCR validation, the heat stress experiment was repeated in its entirety using new individuals, and with four replicates for each temperature and population, for a total of 16 samples. RNA isolation, DNase treatment and RNA purification were performed as above. From each sample, 125 ng of total RNA was reverse-transcribed following the method of Balcells et al. (2011), with one minor modification. Briefly, reverse transcription reactions were performed in a final volume of 10  $\mu$ l containing 1 $\times$  M-MuLV reverse transcriptase buffer (New England Biolabs), 0.1 mM ATP, 0.1 mM of each dNTP, 1  $\mu$ M of RT-primer, 100 U of M-MuLV reverse transcriptase (New England Biolabs) and 1 U of poly(A) polymerase (New England Biolabs). Reactions were incubated at 42°C for 1 hr followed by enzyme inactivation at 95°C for 5 min and were then diluted fivefold with nuclease-free water. The combination of ATP and poly(A) polymerase adds a poly(A) tail to miRNAs, while the use of the oligo (dT)<sub>15</sub> RT-primer allows for the synthesis of first strand cDNA from both mRNA and miRNA molecules, allowing any transcript to be quantified. For each of the 16

samples, another aliquot of RNA was used in a no-enzyme control amplification to confirm the absence of DNA contamination.

Real-time PCRs were performed in 15  $\mu$ l containing 1X iTaq Universal SYBR Green Supermix (Bio-Rad), 0.25  $\mu$ M of each primer and 3  $\mu$ l of cDNA and were run in a CFX96 system (Bio-Rad), with thermal profile as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s and an annealing temperature for 30 s. The PCR product was then assessed by melting curve analysis to check for the presence of a single amplicon. For each primer pair, we determined the best annealing temperature by performing gradient PCRs with a single sample tested at varying annealing temperatures. Up to three different primer pair designs were tested for each of the four loci (Supporting information Table S2). We also optimized PCR conditions of two stable reference genes (Ribosomal Protein P1: *RPLP1*, and serine/threonine-protein phosphatase 2A: *Pp2A*) to be used for normalization. Primer efficiencies were assessed by performing qPCRs of a dilution series of a pool of cDNA templates, in duplicate. Efficiencies were determined to be within the range recommended for quantitative PCR (97%–104.9%, Supporting information Figure S3; Bustin et al., 2009; Schmittgen & Livak, 2008). Finally, working primer pairs and conditions were used to assay the 16 experimental replicates along with no-template controls, with all samples run in technical duplicates.

For each sample, technical duplicates for each locus were averaged and the geometric mean of *RPLP1* and *Pp2A* was used for normalization. The normalized expression for each miRNA gene was calculated as  $2^{-\Delta C_t}$ , where  $\Delta C_t = C_{t_{\text{miRNA of interest}}} - C_{t_{\text{reference genes}}}$ . Treatment groups were then compared via *t* tests, and fold change estimated relative to the mean of normalized expression of the respective control group.

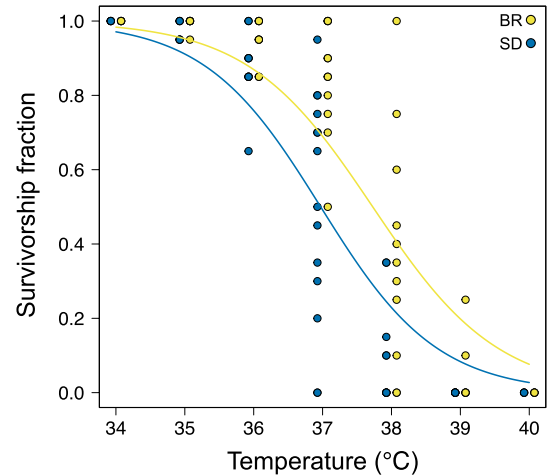
### 3 | RESULTS

#### 3.1 | Population thermotolerance

Consistent with previous studies, BR and SD showed high thermotolerance, with both populations showing zero mortality at 34°C and at least some survivorship at 38°C. Nevertheless, BR replicates survived significantly better at higher temperatures and had a higher LD<sub>50</sub> (LD<sub>50</sub>  $\pm$  SE: BR: 37.7  $\pm$  0.05°C; SD: 36.9  $\pm$  0.04°C; Figure 1). All SD copepods died at 39°C, while BR had some survivorship, albeit not significantly higher than SD (Wilcoxon rank sum test,  $p = 0.139$ ). BR showed significantly higher survivorship at 38°C ( $p = 0.028$ ) and 37°C ( $p = 0.0027$ ).

#### 3.2 | Identification of miRNAs in *T. californicus*

The 12 miRNA-seq samples averaged 26.6 million reads passing quality and length filters (range: 21–37 million; supporting information Table S1). miRDeep2 analysis was performed on BR and SD populations separately (1 heat-stressed and 1 control for each). Naming of miRNAs was standardized between populations based on chromosomal location of precursor sequences. Ultimately, across both populations miRDeep2 analysis predicted 148 putative miRNAs after multiple stringency filters (Supporting

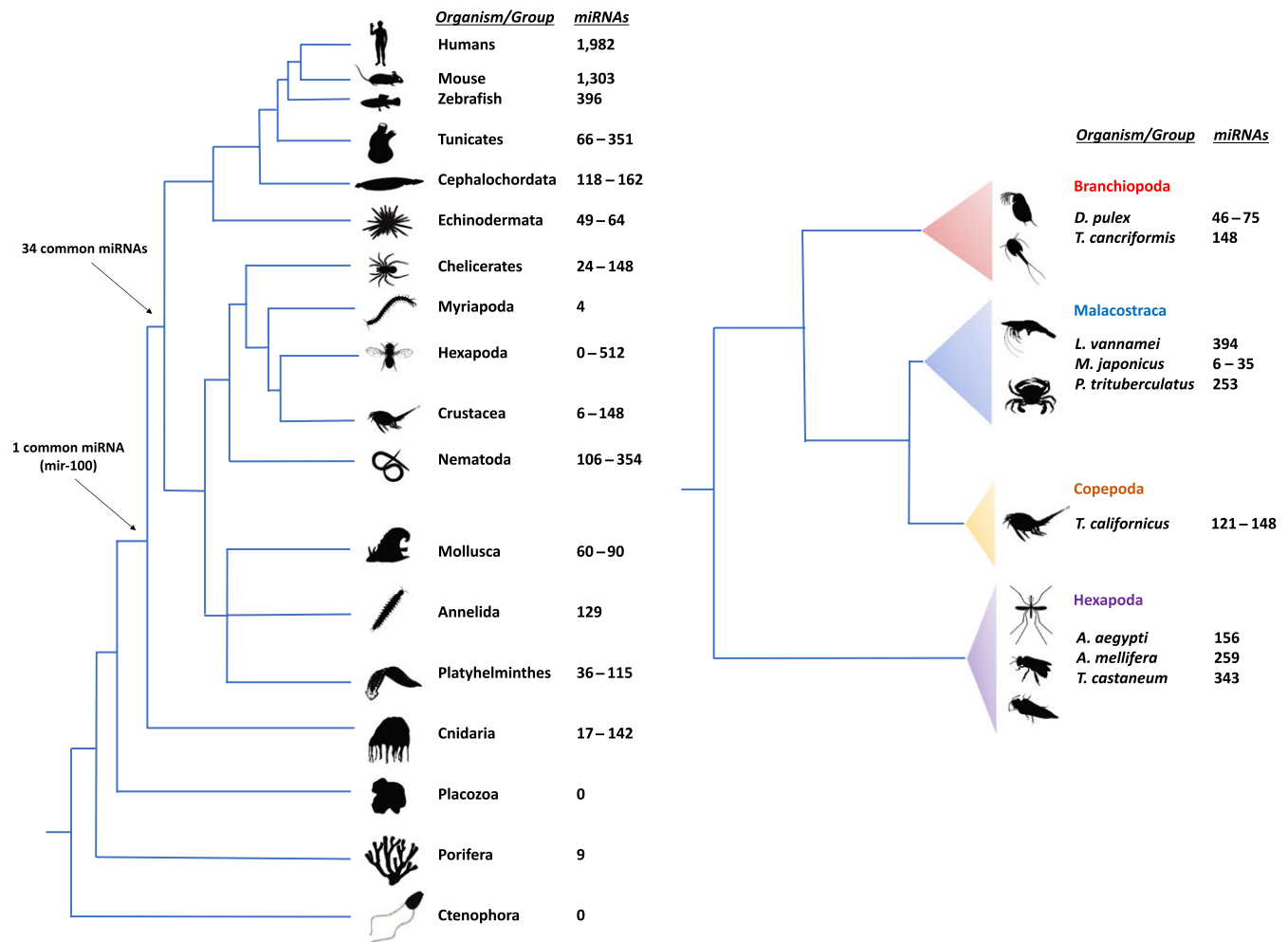


**FIGURE 1** Thermotolerance of two *Tigriopus californicus* populations. Survivorship was assessed three days after a one-hour immersion in target temperatures. For visualization, points from San Diego (SD) and Bird Rock (BR) were shifted horizontally, but the model lines follow the original data. Points from several replicates within a population overlap completely and hence are not visible. Number of replicates at each respective temperature for BR are  $n = 4, 6, 8, 11, 11, 6, 4$ , and for SD are  $n = 4, 6, 10, 14, 10, 7, 4$

information Table S3). The majority of the miRNAs identified were “novel” ( $n = 111$ ), meaning they passed all stringency filters, but did not match any mature miRNAs currently available on miRBase. There were a total of 33 previously identified miRNA classes, although there seem to be *T. californicus*-specific duplications of miR-1175-3p, miR-08, miR-2b and miR-1175, for a total of 37 matches to miRBase. For the SD population, a total of 121 putative miRNAs were identified, including 36 miRNAs with sequence homology to known miRNAs in miRBase and 85 novel miRNAs, of which 24 were SD-specific (i.e., not annotated in BR). For the BR population, a total of 121 putative miRNAs were identified, including 34 previously identified miRNAs and 87 novel miRNAs, of which 26 were BR-specific. The number of miRNA loci identified by this study is within the range of other protostomes, with a range of 60–238 miRNAs (Berezikov, 2011). Although our predicted number of miRNAs is higher than that of the two crustaceans on miRBase, previously published estimates of miRNA repertoires within Pancrustacea show our estimates are well within expectations (Figure 2), including *Litopenaeus vannamei*, *Portunus trituberculatus*, *Daphnia pulex*, *Daphnia magna*, *Triops cancriformis* and *Marsupenaeus japonicus* (Chen, McKinney, Nichols, & Sepúlveda, 2014; Hearn et al., 2018; Ikeda et al., 2015; Meng, Zhang, Li, & Liu, 2018; Ruan et al., 2011; Xi et al., 2015). In addition, across the two populations, all 33 that matched a previously identified miRNA included miRNAs that are conserved in Metazoans, Eukaryotes, Bilaterians, Protostomes and Arthropods, based on miRbase reports (Supporting information Table S3).

The miRNAs characterized were found in various regions of the genome, with 50% found in intergenic regions, and 50% found within some portion of an annotated gene. Of those located





**FIGURE 2** Distribution of miRNA genes across Metazoa (left) and Pancrustacea, where *Tigriopus californicus* is placed (right). Metazoan miRNA counts are from miRbase, while Pancrustacean counts use miRbase and counts from other sources (Chen et al., 2014; Ikeda et al., 2015; Meng et al., 2018; Ruan et al., 2011; Xi et al., 2015). Branch lengths in the cladogram are not to scale. Silhouette images are from Phylopic (CC BY-SA 3.0)

**TABLE 1** Genomic locations of identified miRNAs in *Tigriopus californicus*

Category	Number of miRNAs	% of total miRNAs	% of genic miRNAs
Intergenic	74	50	–
Genic	74	50	–
In exon	16	10.8	21.6
In intron	54	36.5	73.0
In UTR	4	2.7	5.4

Note. UTR: untranslated region, 5' or 3'

within a gene, 21.6% overlapped with an exon, 73% were in intronic regions, and 5.4% were within UTRs (3'UTR and 5'UTR). The majority of novel miRNAs were located within gene regions, mostly in introns (55.8%; Table 1; Supporting information Table S4). Our results match previous patterns of miRNA locations, with most miRNAs residing in intergenic regions. In addition, those that appear within gene regions tend to reside in introns, although this varies widely among surveyed organisms (Campo-Paysaa, Sémon, Cameron, Peterson, & Schubert, 2011; Isik, Korswagen, & Berezikov, 2010; Nozawa, Miura, & Nei, 2010;

Paczynska, Grzemeski, & Szydlowski, 2015; Rodriguez, Griffiths-Jones, Ashurst, & Bradley, 2004).

### 3.3 | Identification of miRNA associated with thermal stress

Differential expression analyses revealed only one miRNA, tcal-mir-novel-81-3p, to be significantly changing in response to thermal stress in both populations (FDR < 5%). This locus exhibited downregulation of 4.3-fold in SD ( $\log_2$  fold-change = -2.1; Supporting information

Table S5; Figure 3) and 2.5-fold in BR ( $\log_2$  fold change =  $-1.35$ ; Supporting information Table S6; Figure 3). However, the BR population showed three additional novel miRNAs responsive to thermal stress (tcal-mir-novel-39-3p, tcal-mir-novel-40-3p, tcal-mir-novel-50-3p; Supporting information Figure S4), all of which were upregulated by  $\sim 2$ -fold in the heat-stressed treatments. The mature sequence of tcal-mir-novel-39-3p and tcal-mir-novel-40-3p differs by only one nucleotide at the terminal of the 3' end, but they are distinct loci (tcal-mir-novel-39-3p is located on chromosome 6 while tcal-mir-novel-40-3p is on chromosome 4; Supporting information Figure S4; Table S3). Moreover, tcal-mir-novel-50-3p was annotated only in the BR population and thus is ostensibly not present in the SD population. To assess whether this miRNA was excluded from SD due to high stringency during annotation, we examined the full mirDeep2 results for SD, including those with low annotation scores, and searched for potential miRNAs with the same genomic coordinates as the BR version. No potential candidates were found in mirDeep2 analyses using either heat stress or control SD miRNA libraries. Using the BR sequence for this miRNA, we searched the

SD genome and found an exact match for the full precursor miRNA, even though mirDeep2 did not detect it as an active miRNA using SD reads. When differential expression analysis was performed using tcal-mir-novel-50-3p as reference for mapping of SD reads, it found substantially fewer matched reads and was not significantly differentially expressed between control and temperature stress replicates in the SD population.

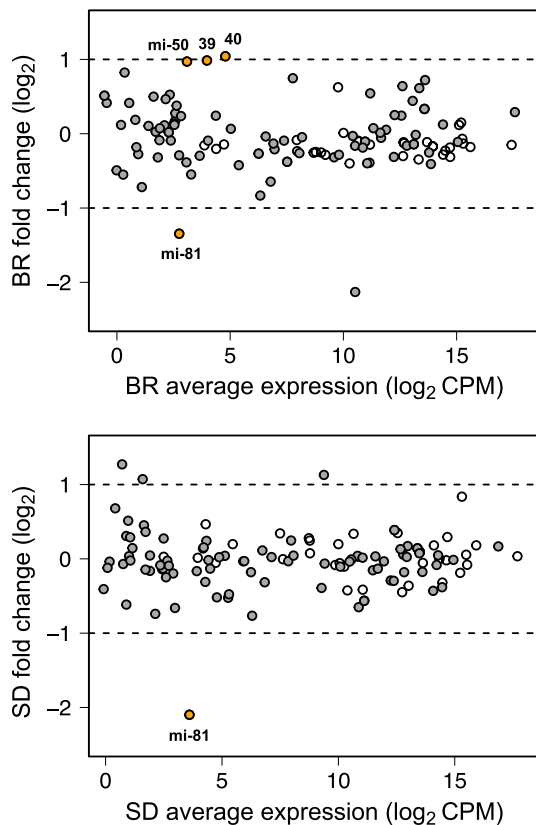
In regard to genome location, both tcal-mir-novel-81-3p and tcal-mir-novel-50-3p are intronic, while tcal-mir-novel-39-3p and tcal-mir-novel-40-3p are intergenic (Table S4).

### 3.4 | qPCR results

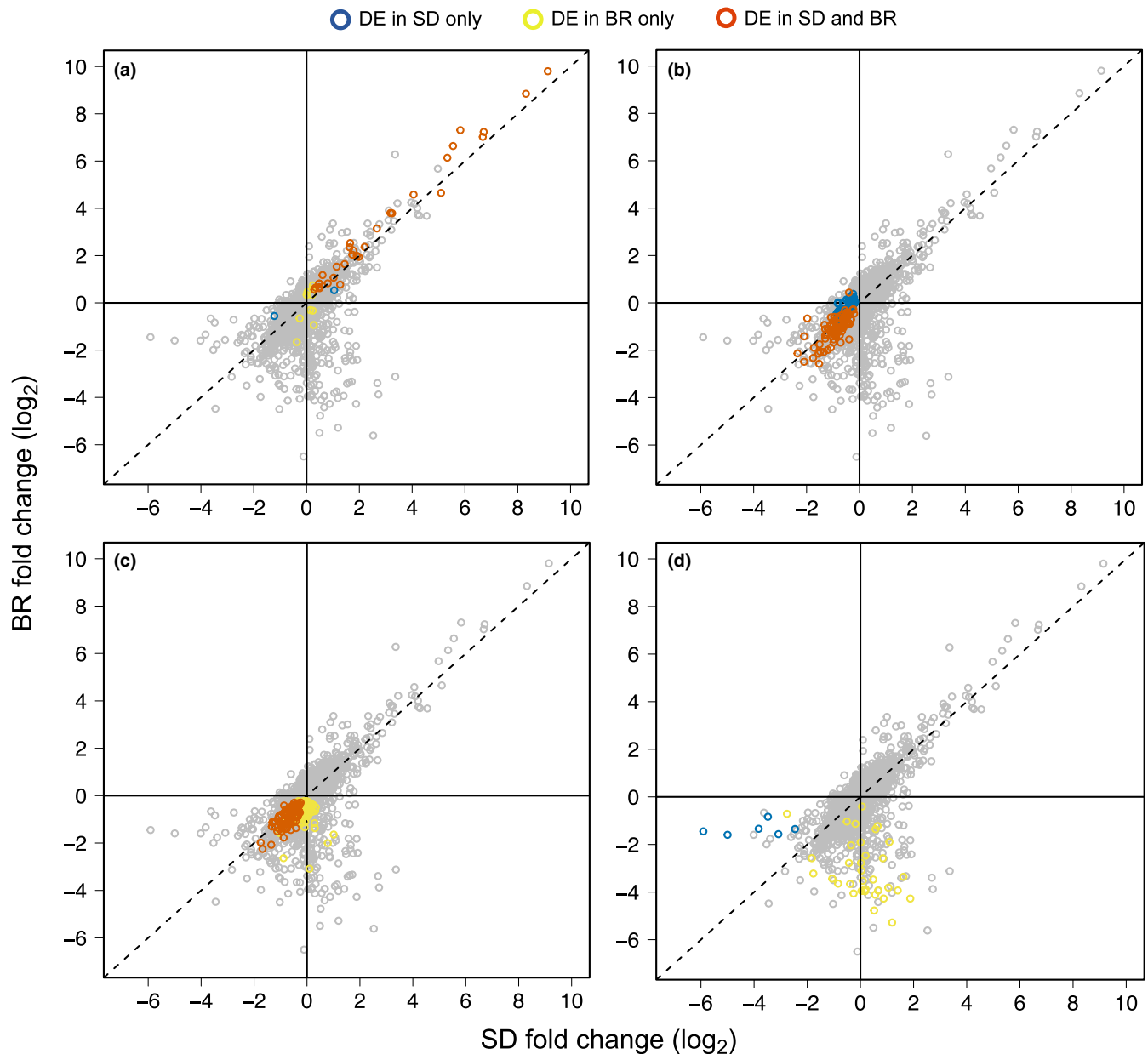
We successfully amplified two miRNA loci (tcal-mir-novel-40-3p and tcal-mir-novel-81-3p) by RT-qPCR, but failed to amplify single products from tcal-mir-novel-39-3p and tcal-mir-novel-50-3p despite multiple primer designs and optimization attempts (Table S2). Specific amplification of mature miRNAs is known to be difficult due to their short length and limited opportunity for primer design. Nonetheless, we were able to routinely amplify two of the four putatively heat-responsive miRNAs from this study, and we repeated the heat stress experiment with new individuals in order to validate the transcriptional changes of these loci. Results from the RT-qPCR were concordant with those from small RNA-seq, with tcal-mir-novel-40-3p being significantly upregulated only in the BR population, while tcal-mir-novel-81-3p was significantly downregulated in both populations, yet to lower levels in SD (Supporting information Figure S5).

### 3.5 | Differential gene expression

Across the 12 mRNA library samples, we obtained an average of 31.8 million after quality and length filters (range: 21.8–49.9; Table S1). Mapping with Salmon resulted in an average of 19.6 million reads per replicate mapped for SD and 17.6 million reads per replicate mapped for BR. A total of 11,466 SD genes were examined for differential expression between control and heat-stressed treatments after passing minimum filter of three reads per million mapped in edgeR. This analysis detected 1,768 differentially expressed (DE) genes (FDR <5%), with 819 being upregulated during heat stress and 949 downregulated (Supporting information Table S7). For the BR population, this analysis revealed over twice as many DE genes (3,848), even as fewer genes in total passed minimum mapping filter (10,944). Among these, 1,888 were upregulated while 1,960 were downregulated (Supporting information Table S8). The majority of DE genes (81%) in both populations changed by modest amounts (1.12- to 1.9-fold change, in either direction). Using a threshold of fold change of 2, the number of DE genes decreased substantially but BR still showed over twice as many as SD (up/down, BR: 198/518, SD: 160/175). Although a higher number of genes tended to be downregulated (at any fold change cut-off), the highest magnitudes of expression change occurred among upregulated genes in both populations (Supporting information Tables S7 and S8, Figure 4, Supporting information Figure S6).



**FIGURE 3** Transcription levels of microRNA in *Tigriopus californicus*. Fold change values are for the comparison between heat-stressed (33°C) and control (20°C) treatments, averaged across replicates. Expression levels are estimated by counts per million (CPM) reads mapped, normalized via the trimmed mean of M-values (TMM) method. Dashed lines denote  $\log_2$  fold change of 1 in the up- or downregulation directions. Open circles: miRNA with homology in miRBase. Closed circles: miRNA putatively novel in *T. californicus*. Orange circles: novel miRNAs that were differentially expressed between treatments. BR: Bird Rock; SD: San Diego



**FIGURE 4** Comparison of fold change in expression due to heat stress in two populations of *Tigriopus californicus*. Plotted are all differentially expressed (DE) genes that were statistically up- or downregulated in at least one of the two populations. Each panel highlights a specific functional group of interest. (a) *Heat-shock protein (Hsp)* genes, including *DnaJ* genes; (b) nervous system development and regulation; (c) ion transport; (d) cuticle proteins. SD: San Diego; BR: Bird Rock. Dashed line has a slope of 1 to depict where the two populations are equal

A total of 1,117 genes showed significant differential expression in both populations (Supporting information Figure S6), and 99% (1,106) of these changed in the same direction between populations. Not surprisingly, heat-shock protein genes (*Hsps*) were among the most highly inducible genes, with nine *Hsp* genes among the top 10 upregulated genes in both populations (Supporting information Tables S7 and S8). Concordantly, the lists of upregulated genes were highly enriched for GO terms associated with the heat-shock response, such as "protein folding," "response to heat," "response to stress" (Supporting information Tables S9 and S10). This was especially true when only genes with fold change  $\geq 2$  were considered

(Supporting information Tables S11 and S12). Most *Hsps* showed relatively modest responses (1.5 to 10x upregulation), but among genes of the small HSP (*Hsp20*) and *Hsp70* families, some increased 300- to 900-fold levels during heat stress relative to controls (Figure 4a; Supporting information Tables S7 and S8).

Despite broad similarities when comparing the small set of highly upregulated genes, SD and BR showed striking differences across several levels. We observed a high number of genes that were differentially expressed in only one population, with 651 in SD and 2,731 in BR. In addition, among the 1,106 genes that changed in both populations, BR consistently showed a greater magnitude of change than



SD in both up- ( $n = 548$ , paired Wilcoxon signed rank test,  $p < 10^{-16}$ ) and downregulation ( $n = 558$ ,  $p < 10^{-16}$ ) (Supporting information Figure S6). This pattern was true even within specific groups, such as *Hsps* ( $n = 31$ ,  $p = 6.1 \times 10^{-6}$ ; Figure 4a). The populations also differed greatly in the number of functional categories among up- and downregulated gene sets, as reflected by Fisher exact tests for overrepresentation of GO terms at FDR <5% (Supporting information Tables S9, S10, S13, S14). While “stress response” and related terms were among the top overrepresented categories within SD upregulated genes (Supporting information Table S9), these terms ranked much lower in BR, for which several DNA and RNA “metabolic processes” were most enriched (Supporting information Table S10). BR had twice as many overrepresented GO terms among upregulated genes (576) compared to SD (291), but among downregulated genes, there were 5× more GO terms in SD (492) compared to BR (94). We compared these numbers with regard to how many functional terms SD and BR shared. A total of 661 unique GO terms were overrepresented within upregulated genes, 205 of which were shared between the two populations. Within downregulated genes, only 39 were shared from a list of 545 unique terms. These ratios were significantly different (Fisher’s exact test,  $p < 10^{-15}$ ). Even when only the most downregulated genes were considered (fold change  $\leq -2$ ), major differences in GO term enrichment remained (Supporting information Tables S15 and S16). This suggests that SD and BR differed substantially in both the number and function of genes that were differentially expressed and that this divergence is especially strong for the types of cellular processes that were downregulated during heat stress. Top overrepresented categories among downregulated genes in SD but not BR include neuron differentiation and development (Figure 4b; Supporting information Table S13), while top terms in the BR list but not in SD include ion transport (Figure 4c) and cuticle proteins (Figure 4d) (Supporting information Table S14).

### 3.6 | Linking miRNAs to their putative targets

To determine the potential function of the four putative thermal stress-responsive miRNAs, mRNA targets were predicted using miRanda, against the 10,297 genes which had an annotated 3’UTR in the *T. californicus* genome. For both tcal-mir-novel-39-3p and tcal-mir-novel-40-3p, due to the extreme similarity in their mature miRNA sequence, miRanda

predicted the same potential binding sites, with 392 genes classified as having a potential binding site match. For tcal-mir-novel-81-3p and tcal-mir-novel-50-3p, miRanda detected 289 and 329 gene matches, respectively (Supporting information Table S17). The overall distribution of gene targets was similar among the four miRNA, with the majority of targets showing no change in expression during heat stress, and among the subset of DE genes, expression change occurred at similar frequency in both directions (Supporting information Figure S7).

We first assessed the breadth of functional categories encompassed by all potential gene targets of these four miRNAs. We found no significant GO terms at FDR of 0.05. However, not all genes currently annotated in the genome (15,646) had 3’ UTRs available, and hence, the reference subset for the GO term enrichment is a smaller group (10,297). Thus, it is possible that a lack of overrepresented GO terms is not indicative of a lack of commonality among putative miRNA targets, but instead may be due to the makeup of the reference list during the analysis. When tested against the full gene list, tcal-mir-novel-81-3p and tcal-mir-novel-50-3p did show significantly overrepresented GO terms, while tcal-mir-novel-39-3p and tcal-mir-novel-40-3p did not. For tcal-mir-novel-81-3p, the GO terms encompassed categories associated with nervous system/neuronal differentiation, and apoptosis, in addition to a general response to stimulus and stress (Table 2; Supporting information Table S18). Finally, tcal-mir-novel-50-3p had terms that were largely associated with signalling/communication, response to a stimulus and various metabolic/catabolic processes (i.e., cellular, protein, nitrogen) (Table 2; Supporting information Table S19).

Because miRNAs regulate mRNA levels through their degradation, direct mRNA targets can be expected to show direction of expression that is reciprocal to that of their binding miRNA. We assessed the composition of the putative miRNA targets by filtering for those that were differentially expressed in the expected direction (i.e., upregulated miRNA-downregulated mRNA). For the upregulated tcal-mir-novel-39-3p and tcal-mir-novel-40-3p, 45 of the 392 potential gene targets (11.5%) were significantly downregulated between control and heat-stressed treatments. For the downregulated tcal-mir-novel-81-3p, 22 miRanda matches were significantly upregulated in SD (7.6%), while 52 were significantly upregulated in BR (17.9%). Finally, of the genes detected as potential targets for tcal-mir-novel-50-3p (upregulated in BR), 54 (16.4%) were significantly downregulated (Supporting information Figure S7, Table 3).

**TABLE 2** Summary information for putative miRNA targets that were differentially expressed in either direction

miRNA	No. of predicted targets	No. of targets differentially expressed (SD)	No. of targets differentially expressed (BR)	Significantly overrepresented GO terms among differentially expressed targets
Tcal-mir-novel-81-3p	290	49 (FDR < 0.05) 71 (FDR < 0.1)	88 (FDR < 0.05) 130 (FDR < 0.1)	Response to a stress, metabolic processes, nervous system/neuronal differentiation, apoptosis
Tcal-mir-novel-39/40-3p	392	–	109 (FDR < 0.05) 160 (FDR < 0.1)	None
Tcal-mir-novel-50-3p	329	–	94 (FDR < 0.05) 140 (FDR < 0.1)	Signalling/communication, response to a stimulus, metabolic processes

Note. Gene Ontology (GO) terms associated with these targets were detected at FDR of 0.1 (Supporting information Tables S14–S17).

When these gene sets were assayed for overrepresentation of functional GO terms, none showed enrichment at FDR of 0.05, likely due to the small number of genes in each list reducing statistical power. However, a more liberal assessment ( $p$ -value <0.01) show some enriched GO term categories. Putative miRanda targets for *tcal-mir-novel-81-3p* that were upregulated in the SD population included steroid hormone receptor signalling pathway, and apoptotic signalling pathway, while for the BR population, GO terms included cellular macromolecule metabolic process, regulation of cell cycle and cellular response to stress (Table 3; Supporting information Tables S20 and S21). Both *tcal-mir-novel-39/40-3p* and *tcal-mir-novel-50-3p* were upregulated in the BR population, and their putative targets that were significantly downregulated are enriched for neurotransmitter transport, anion transport, G protein-coupled receptor signalling pathway, amine transport and ion transmembrane transporter activity (Table 3; Supporting information Tables S22 and S23).

Finally, since we obtained matched small RNA and mRNA sequencing libraries, we examined miRNA-mRNA expression correlations for each of the DE miRNAs and their DE targets. All targets that showed reciprocal direction of expression (Table 3) had significant negative Pearson correlations with their respective miRNA across samples ( $r$  range: -0.71 to -0.99; Supporting information Table S17). We also observed similar numbers of positive correlations ( $r$  range: 0.63 to 0.99; Supporting information Table S17), with 59 for *tcal-mir-novel-39/40-3p*, 41 for *tcal-mir-novel-50-3p* and 37 for *tcal-mir-novel-81-3p* in BR, and 28 for *tcal-mir-novel-81-3p* in SD (Supporting information Table S17). A total of 34 DE genes were predicted to be targets of two different miRNAs (counting *tcal-mir-novel-39/40-3p* as one miRNA) and showed significant expression correlations to both miRNAs. We used VisANT (Hu, Mellor, Wu, & DeLisi, 2004) to visualize the miRNA-mRNA network for BR, including only links with  $|r| \geq 0.90$  (Figure 5).

## 4 | DISCUSSION

This study characterized fine-scale population-level differences in transcriptional response to temperature stress for both mRNA

and miRNAs in *T. californicus*. The two populations examined (SD and BR) are located only 8 km apart and hence experience broadly the same temperature regime. These populations were of particular interest because Pereira et al. (2014) showed that SDxBR crosses can generate recombinant hybrids that are substantially better at surviving acute temperature stress relative to the parental stocks. The lack of gene flow between SD and BR has allowed them to follow independent paths to adaptation to daily high temperatures. We found strong evidence that SD and BR have evolved population-specific regulatory networks for stress response, including mRNA and miRNAs, that can serve as raw material for transgressive segregation.

### 4.1 | Divergence in transcriptional response

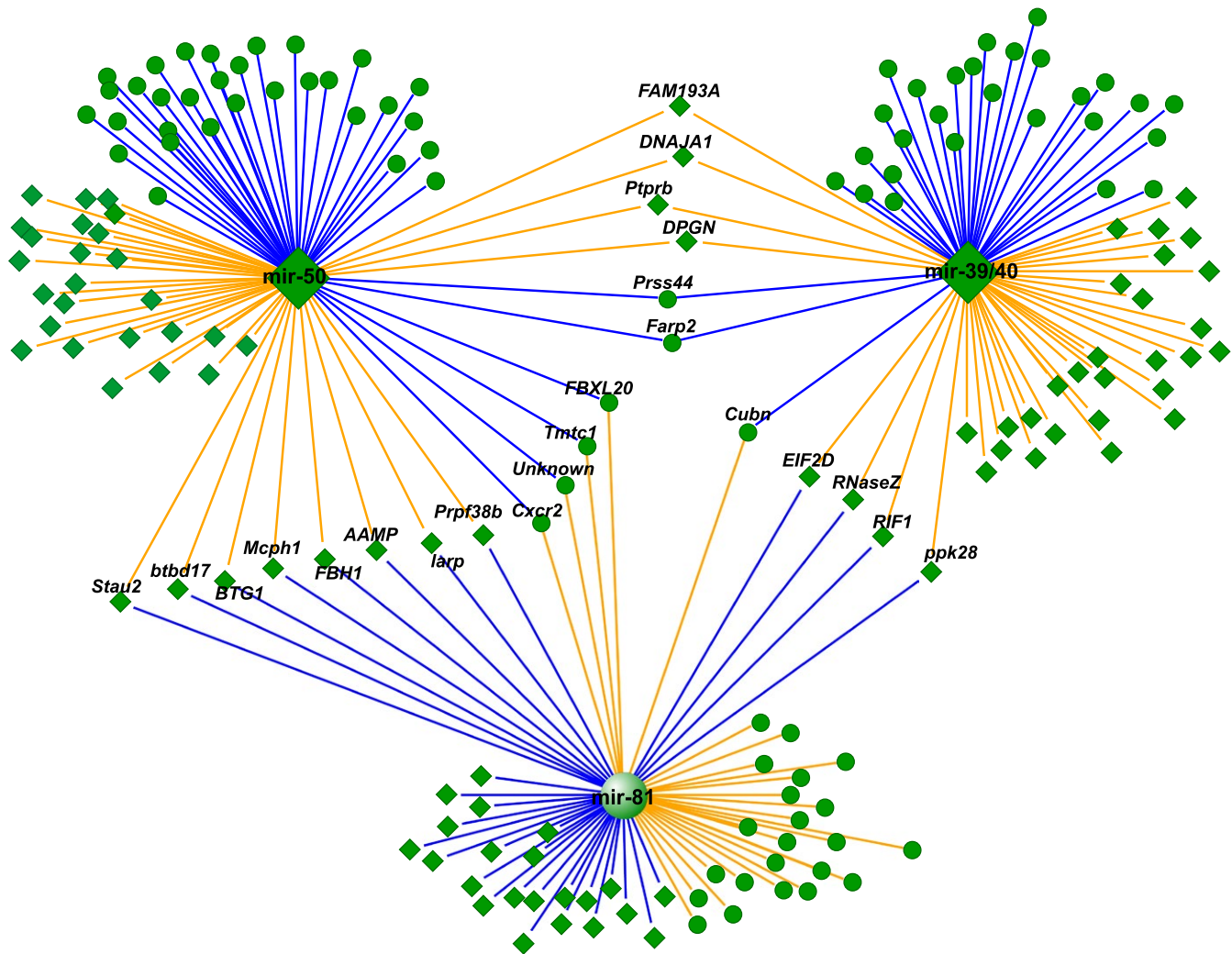
Genes that directly function as molecular chaperones in response to temperature stress (e.g., *Hsps*) were the most commonly upregulated in both populations examined, and some genes showed induction as high as ~800-fold. Among these genes, however, one of the populations (BR) showed a consistently higher magnitude of upregulation. Since BR is more thermotolerant than SD, this pattern of expression is consistent with previous finding from Schoville et al. (2012), which reported that the more thermotolerant population (SD in their study) showed higher induction of *Hsp* genes compared to a less tolerant population from Northern California. Examining populations of even higher and lower temperature sensitivity is warranted to assess this emerging pattern.

Despite a substantial overlap in number of differentially expressed genes (1,117), there were enough population-specific patterns of expression to result in clear widespread differences in functional divergence. BR had several "metabolism" gene groups that were more overrepresented than the *Hsp*-related "protein folding" genes, suggesting this population has evolved an expanded network of functional pathways to deal with elevated temperature. Functional divergence was especially magnified while examining genes that were downregulated. SD showed bias towards genes involved in the nervous system, while BR

**TABLE 3** Summary information for miRNA targets that were differentially expressed in a reciprocal direction

miRNA	miRNA expression direction	No. of predicted targets	No. of targets differentially expressed	"Top" GO Terms for predicted targets
<i>Tcal-mir-novel-81-3p</i>	SD: Down BR: Down	290	SD: 21 up BR: 51 up	SD: steroid hormone receptor signalling pathway, apoptotic signalling pathway BR: cellular macromolecule metabolic process, regulation of cell cycle, cellular response to stress
<i>Tcal-mir-novel-39/40-3p</i>	SD: no change BR: Up	392	SD: N/A BR: 50 down	BR: Neurotransmitter transport, anion transport, synaptic signalling, ion transmembrane transporter activity
<i>Tcal-mir-novel-50-3p</i>	SD: not expressed BR: Up	329	SD: N/A BR: 53 down	BR: G protein receptor signalling pathway, amine transport

Notes. Differential gene expression was assessed at FDR of 0.05. Gene Ontology (GO) terms associated with these targets were detected at  $p$ -value < 0.01 (Supporting information Tables S16–S19). N/A: Not applicable.



**FIGURE 5** Partial predicted interaction network between temperature-responsive miRNAs and mRNAs. The three large nodes depict the differentially expressed miRNAs in the BR population, while all other nodes are their target mRNAs. Diamond-shaped nodes show upregulated loci; circular nodes show downregulated loci. Blue edges depict negative correlations; orange edges depict positive correlations. All miRNA-mRNA connections shown had computational predictions of binding as well as significant Pearson correlation across replicates. For ease of visualization, shown are only edges with Pearson  $r \geq 0.90$ , and gene abbreviations only for nodes connecting two modules. See Supporting information Table S17 for a complete list of predicted interactions

had more genes involved in ion transport and cuticle proteins. Broadly, these functional categories have been shown to be involved in a response to temperature stress. Specifically, neural elements are highly sensitive to temperature changes, including effects on action potential, conduction and other synaptic parameters (Janssen, 1992; Robertson, 2004; Robertson & Money, 2012). Ultimately, protection of neural signalling from temperature stress is manifested in the upregulation of protein chaperones and modulation of the conductance of potassium.

The cuticle of Pancrustacea has also shown to play a role in ion transport across membranes in the presence of temperature stress, though such examples are in cold-hardened insects (Clark & Worland, 2008; Qin, Neal, Robertson, Westwood, & Walker, 2005). Nonetheless, research suggests that genes involved in phospholipid composition of membranes (like the cuticle) are frequently involved in a molecular

response to a temperature stress, specifically involving osmotic balance/ion homeostasis, as well as nervous system functioning (Overgaard & MacMillan, 2017). Divergence in the use of cuticle protein genes was particularly striking in that BR downregulated 34 such genes, while SD only 6, with no overlap between them (Figure 4d; Supporting information Tables S7 and S8). In a recent study of salinity stress in *T. californicus*, DeBiaise, Kawji, and Kelly (2018) detected overrepresentation of genes associated with ion transport and cuticle. Specifically, ion transport genes were downregulated during high-salinity stress, indicating a likely shared mechanism with response to temperature stress. Cuticle genes, however, were observed to be significantly upregulated in response to low-salinity (DeBiaise et al., 2018). The functional explanations for these differences between populations and across different stressors are not known but could provide a framework for understanding adaptations in this physiologically hardy species.

## 4.2 | miRNAs and temperature stress

Relative to curated databases of animal miRNAs, most *T. californicus* miRNAs annotated in this study were novel. Some miRNAs appeared to be population-specific, although this was likely due to differences in expression, which may have been too low to warrant annotation via the pipeline, yet still present within the genome. We identified one miRNA that was significantly heat-responsive in both SD and BR populations (tcal-mir-novel-81-3p), though to different degrees (fourfold in SD and twofold in BR). Since tcal-mir-novel-81-3p was downregulated, it would be expected to release its repression on their targets, resulting in the potential for upregulation of those targets. The mRNA targets predicted for tcal-mir-novel-81-3p are enriched for functional categories also found among upregulated genes, and which are consistent with the heat-shock response, including protein ubiquitination and cellular response to stress. Thus, this single miRNA appears to be directly involved in repression of these heat-responsive genes under nonstressful temperature conditions, similar to a recent finding in the coral *Acropora digitifera* (Gajigan & Conaco, 2017). We expect tcal-mir-novel-81-3p to be a conserved regulatory component of the heat-shock response across *T. californicus* as a whole and will examine its expression in additional populations, in the future.

Three other miRNAs were detected as heat-responsive only in BR. This exclusivity was further emphasized by the fact the one of these miRNAs (tcal-mir-novel-50-3p) was annotated only in BR, even though a similarly high number of SD reads were used during miRD-eep2 modelling. Alignment of this BR miRNA with the SD genome revealed that the sequence of the full miRNA precursor is found in the SD genome and on the same chromosomal location. This suggests that SD may lack the machinery to activate or transcribe this molecule, or at least that it does not do so in either of the experimental conditions examined in this study. Moreover, we hypothesize that, since these three miRNAs (tcal-mir-novel-39-3p, -40-3p, and -50-3p) were differentially transcribed only in BR, their mRNA targets should be disproportionately affected in BR relative to SD. After accounting for the difference in total number of DE genes between SD and BR, we found that, among tcal-mir-novel-39/40-3p targets (which are identical), the proportion of genes that were DE only in BR ( $n = 74$ ) was not significantly higher than the proportion DE only in SD (24) (Fisher's exact test,  $p = 0.154$ ). We did find, however, that among targets of tcal-mir-novel-50-3p, the proportion of genes that were DE only in BR ( $n = 55$ ) was significantly higher than that in SD (9) (Fisher's exact test,  $p = 0.0017$ ). Conversely, the proportions of BR-only and SD-only DE genes were not different among targets of tcal-mir-novel-81-3p (BR: 60, SD: 24,  $p = 0.636$ ), which is consistent with this association, as this miRNA was differentially expressed in both populations. These patterns suggest that, at least tcal-mir-novel-50-3p and tcal-mir-novel-81-3p are regulators of heat-responsive genes, and are also associated with differences in transcriptional response between the two populations.

Current understanding of miRNA proximate mechanisms in animals indicates that it functions by binding to target mRNA

to direct its degradation or to prevent its translation, and hence that they function as repressors. When certain genes require increased transcription during periods of cellular stress, their repressor miRNAs are downregulated, and vice versa. Yet, studies have shown that as a miRNA is upregulated, its target mRNAs may also show transcription increase, highlighting the complexity of miRNA-mRNA regulatory dynamics (Laxman et al., 2015; Nunez-Iglesias, Liu, Morgan, Finch, & Zhou, 2010; Vasudevan, Tong, & Steitz, 2007). Such counter-intuitive positive correlations may be the result of downstream feedbacks (Martinez, Ow, & Barrasa, 2008). Alternatively, miRNAs may be upregulated in order to attenuate the levels of highly induced genes, thus better controlling the number of mRNA copies destined for translation (Herranz & Cohen, 2010; Schmiedel et al., 2015). Attenuation of such a transcriptional response may be the result of selection pressure against an increased response to an environmental stressor that is ultimately counterproductive (i.e., maladaptive, as per Ghalambor, McKay, Carroll, & Reznick, 2007). For example, initial responses to a novel environmental stressor have the potential to negatively impact survival/fitness over an extended period of time; thus, populations would benefit from the ability to attenuate the response, or suppress such a reaction long-term, through compensatory mechanisms (Grether, 2005; Lande, 2009). This situation would be the case for both the SD and BR populations upon their initial colonization and likely resulted in different molecular mechanisms to temperature stress, especially at the level of miRNAs.

Specifically, we argue that the BR population of *T. californicus*, relative to the SD population, evolved this additional role for miRNAs to blunt elements of the unsuitable response in order to achieve thermotolerance. In the case of *Tigriopus*, this may be manifested in the fact that BR expresses twice as many genes as SD during stress, and additional miRNAs might be needed to control this response. It has been suggested that the power of miRNAs is in the cumulative weak repression of many targets, which is thought to ultimately stabilize gene regulatory networks (Zhao, Shen, Tang, & Wu, 2017). All together, the three upregulated miRNAs in BR may be providing an additive effect on stabilizing the physiological response to thermal stress. Based on computational predictions of binding and correlation of expression levels, the miRNA-mRNA network provides a hypothesis for potential interactions between different regulatory levels and highlights how certain genes require multiple interactions for appropriate modulation. These results further highlight varying avenues that thermally adapted populations of *T. californicus* may have utilized, with SD and BR converging on similar levels of thermotolerance through different mechanisms.

Characterization of miRNA sequence and transcription via RNA-seq provides exciting opportunities to better examine the complex reality of regulatory networks. Nonetheless, initial analyses of miRNA-mRNA networks are difficult and come with caveats. First, all miRNAs are known to regulate target transcripts with some level of seed matching to the mRNA. Animals exhibit a loose complementarity of seed to



target region; however, because binding is incomplete, a combination of elements, including transcription factors, are required to effectively co-regulate and suppress expression (Arora, Rana, Chhabra, Jaiswal, & Rani, 2013; Bartel, 2009; Berezikov, 2011; Krek et al., 2005). Second, although *in silico* target prediction methods are generally effective in identifying canonical binding sites for miRNAs, there is a fair degree of uncertainty, with most methods shown to detect ~50% of known miRNA targets (Alexiou, Maragkakis, Papadopoulos, Reczko, & Hatzigeorgiou, 2009; Baek et al., 2008; Liu, Li, & Cairns, 2012). This is partially driven by the commonly accepted mechanism of miRNA targeting in animals, involving site complementarity of the 3' UTR. Yet, increasing evidence demonstrates that targeting can also be mediated through sites other than the 3'UTR (Hausser & Zavolan, 2014; Pasquinelli, 2012; Thomson, Bracken, & Goodall, 2011). Finally, some miRNAs may function primarily as translational repressors and not necessarily direct mRNA degradation, resulting in little impact on standing levels of mRNA (Bazzini, Lee, & Giraldez, 2012; Djuranovic, Nahvi, & Green, 2012; Wilczynska & Bushell, 2015). Ultimately, experimentation is necessary to adequately identify genuine miRNA targets and determine their regulatory dynamics, perhaps via high-throughput sequencing—cross-linking immunoprecipitation (HITS-CLIP) or other similar methods (Baumgarten et al., 2018; Chi, Zang, Mele, & Darnell, 2009; Hafner et al., 2010).

### 4.3 | Conclusions

Overall, our results suggest that although a majority of the transcriptional response in these two geographically close populations is governed by the canonical heat-shock response, substantial divergence has occurred in their mechanisms of acute heat stress tolerance. The two populations examined are relatively tolerant to elevated temperatures, but they differ significantly in the number of genes involved, the magnitude of change in commonly used genes, and in the number of miRNAs activated during stress. By targeting multiple elements of a regulatory network, each miRNA has the potential to provide an additional level of control of gene and protein expression levels. The more pronounced transcriptional response of BR copepods may require additional levels of control to modulate mRNA quantity destined for translation, and we hypothesize that the use of additional miRNA loci, compared to SD, plays this regulatory role. This increased level of network complexity may partially explain BR's superior survivorship. We are currently developing transgressive SDxBR hybrid lines to examine the role of population-specific miRNA expression in generating extreme phenotypes.

### ACKNOWLEDGEMENTS

We wish to thank M. Dasenko for help with Illumina sequencing and S. Renaut and two anonymous reviewers for insightful feedback on earlier versions of the manuscript. This work was supported by a U.S. National Science Foundation grant (no. DEB-1556455) to F.S.B.

### AUTHOR CONTRIBUTIONS

A.M.G. and F.S.B. designed the research, A.M.G. performed the experiments, A.M.G. and F.S.B. analysed the data, and A.M.G. and F.S.B. wrote the manuscript.

### DATA ACCESSIBILITY

Both mRNA and small RNA sequence reads are deposited in the NCBI Sequence Read Archive under BioProject PRJNA503482 (Accession nos SRR8146188- SRR8146211). Scripts associated with analysis are available on GitHub (<https://github.com/amgraham07>).

### ORCID

Allie M. Graham  <https://orcid.org/0000-0001-7404-168X>

Felipe S. Barreto  <https://orcid.org/0000-0002-7949-7747>

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## SUPPORTING INFORMATION

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**How to cite this article:** Graham AM, Barreto FS. Novel microRNAs are associated with population divergence in transcriptional response to thermal stress in an intertidal copepod. *Mol Ecol*. 2019;28:584–599. <https://doi.org/10.1111/mec.14973>