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microRNA expression variation as a potential molecular mechanism contributing to adaptation to hydrogen sulphide

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Abstract

microRNAs (miRNAs) are post-transcriptional regulators of gene expression and can play an important role in modulating organismal development and physiology in response to environmental stress. However, the role of miRNAs in mediating adaptation to diverse environments in natural study systems remains largely unexplored. Here, we characterized miRNAs and their expression in *Poecilia mexicana*, a species of small fish that inhabits both normal streams and extreme environments in the form of springs rich in toxic hydrogen sulphide (H_2S). We found that *P. mexicana* has a similar number of miRNA genes as other teleosts. In addition, we identified a large population of mature miRNAs that were differentially expressed between locally adapted populations in contrasting habitats, indicating that miRNAs may contribute to *P. mexicana* adaptation to sulphidic environments. In silico identification of differentially expressed miRNA-mRNA pairs revealed, in the sulphidic environment, the downregulation of miRNAs predicted to target mRNAs involved in sulphide detoxification and cellular homeostasis, which are pathways essential for life in H_2S-rich springs. In addition, we found that predicted targets of upregulated miRNAs act in the mitochondria (16.6% of predicted annotated targets), which is the main site of H_2S toxicity and detoxification, possibly modulating mitochondrial function. Together, the differential regulation of miRNAs between these natural populations suggests that miRNAs may be involved in H_2S adaptation by promoting functions needed for survival and reducing functions affected by H_2S. This study lays the groundwork for further research to directly demonstrate the role of miRNAs in adaptation to H_2S. Overall, this study provides a critical stepping-stone towards a comprehensive understanding of the regulatory mechanisms underlying the adaptive variation in gene expression in a natural system.

KEYWORDS

differential expression, ecological genomics, hydrogen sulphide, local adaptation, microRNAs, *Poecilia mexicana*

1 | INTRODUCTION

Deciphering the molecular underpinnings of adaptive evolution is a core goal of biology (Barrett & Hoekstra, 2011). Whereas many studies focus on identifying adaptive genetic variation in protein-coding genes (Bustamante et al., 2005), variation in gene expression also plays critical roles in adaptation (Romero et al., 2012; Whitehead & Crawford, 2006). Identifying the mechanisms underlying adaptive variation in gene expression is much less studied, especially in non-model systems exhibiting local adaptation to diverse sources of natural selection (Gibson & Weir, 2005). For one, gene expression is notoriously plastic. It can be altered in cell type-specific ways within short temporal scales and in response to a wide variety of internal (e.g. health or reproductive status) and external (e.g. abiotic or biotic environmental) factors (Bajić & Poyatos, 2012; Cheviron et al., 2008; Dietz & Somero, 1992). In addition, diverse mechanisms are involved in the transcriptional and post-transcriptional regulation of gene expression (Holoch & Moazed, 2015; Romero et al., 2012). For example, gene expression differences among individuals or populations can be caused by changes that modulate transcription, including DNA sequence changes in cis- or trans-acting regulatory elements (Osada et al., 2017; Signor & Nuzhdin, 2018) and epimutations (Gibney & Nolan, 2010), or changes that impact the rate of translation, including factors that impact the stability and longevity of mRNAs and the ability of ribosomes to synthesize proteins (Wu & Belasco, 2008).

One mechanism of post-transcriptional regulation of gene expression is mediated by microRNAs (miRNAs). miRNAs are short (~20–22 base pairs) non-coding RNAs that when incorporated into the RNA-induced silencing complex (RISC) can bind to complementary sequences in mRNA molecules (Bartel, 2018; Carthew & Sontheimer, 2009). miRNA-mRNA interactions, most frequently in the 3′-untranslated regions (3′ UTRs) of the mRNA, often reduce variation in gene expression (Ebert & Sharp, 2012; Schmiedel et al., 2015) and the amount of protein produced from transcribed genes (Valencia-Sanchez et al., 2006). This can happen because miRNA binding directly reduces the effectiveness of ribosomes and hinders translation (Valencia-Sanchez et al., 2006). In addition, miRNAs decrease mRNA stability and longevity because miRNA binding accelerates deadenylation rates and subsequent mRNA breakdown, and the RISC can directly cleave mRNAs and render them non-functional (Bartel, 2018; Jonas & Izaurralde, 2015; Valencia-Sanchez et al., 2006).

As post-transcriptional regulators, miRNAs can play critical roles in modulating cellular identity and organismal function (Christodoulou et al., 2010; Kosik, 2010). Most significantly, the regulation and dysregulation of miRNAs are linked to a wide variety of diseases in humans (Ardekani & Naeini, 2010; Garzon et al., 2009; Mendell & Olson, 2012). As with any other mechanism generating functional variation among individuals, the expression of miRNAs may therefore be a target of natural selection and play a role in generating adaptive trait variation (Barrio et al., 2014; Chen et al., 2012). Although not widely studied in natural study systems, there is increasing evidence for variation in miRNA expression among locally adapted lineages (Franchini et al., 2016, 2019; Rastorguev et al., 2017), and in some instances, evidence that such differential expression has functional ramifications for development and perhaps ultimately organismal fitness (Kapralova et al., 2014). Despite the fact that miRNAs can play an important role in modulating the development and physiology of organisms in response to environmental stress, their role in mediating adaptation to diverse environments in natural study systems remains largely unexplored.

A natural model that has been used to investigate adaptation to physiochemical stress in extreme environments is *Poecilia mexicana*, a small livebearing fish in the family Poeciliidae. The species has a large distribution in Mexico and Central America, inhabiting a variety of freshwater habitats (Alda et al., 2013; Palacios et al., 2016). In southern Mexico in the Río Grijalva basin, the species has colonized extreme environments in the form of caves and springs rich in toxic hydrogen sulphide (H_2S) (Tobler et al., 2008, 2011). H_2S is a gas that is naturally enriched in the groundwater feeding sulphidic springs, and H_2S toxicity stems from its ability to bind to complex IV of the mitochondrial respiratory chain, effectively shutting down the aerobic production of ATP (Bagarinao, 1992; Tobler et al., 2016). Different lineages of *P. mexicana* have independently colonized H_2S-rich springs in different drainages of the Río Grijalva basin, resulting in replicated population pairs in sulphidic and adjacent nonsulphidic habitats (Palacios et al., 2013; Tobler et al., 2011). Adjacent populations in different habitats are locally adapted, with complex phenotypic trait differences and genetic divergence across small spatial scales and in the absence of physical barriers that would prevent fish movement (see Tobler et al., 2018 for a review).

Past studies have shown that gene regulation plays a critical role in adaptation to the extreme environmental conditions in H_2S-rich springs. In replicated populations of *P. mexicana* and other poeciliid species that have colonized similar habitats, genes associated with H_2S detoxification as well as aerobic and anaerobic metabolism have convergent expression profiles in the gills (Greenway et al., 2020; Kelley et al., 2016). Common-garden experiments and laboratory H_2S-exposure experiments have shown that population differences of gene expression in nature are not just the product of plasticity reflecting population-specific exposure histories, but substantial variation in gene expression between populations is a consequence of constitutive expression differences and variation in the inducibility of gene expression in response to H_2S exposure (i.e. population variation in plasticity; Passow, Henpita, et al., 2017). What remains unclear is just how exactly evolution has acted to generate the distinct patterns of gene expression among the locally adapted populations of *P. mexicana*.

In the present study, we hypothesized that miRNAs contribute to *P. mexicana* adaptation to sulphidic environments and that miRNA expression in gills modulates protein pathways important for adaptation to H_2S. We focused on a single population pair of *P. mexicana* to characterize miRNAs in the genome of the species and to test for differential expression of miRNAs between sulphidic and nonsulphidic habitats. Gills were selected because they are in direct contact with H_2S dissolved in the water and are involved in the maintenance of

homeostasis (Evans et al., 2014). In addition to testing for differential expression of miRNAs, we also predicted potential mRNA targets of miRNAs in silico and identified integrated miRNA-mRNA pairs with inverse expression patterns between populations using transcriptomic data. This study provides a critical stepping-stone in the investigation of regulatory mechanisms underlying the adaptive variation in gene expression in a natural system.

2 | MATERIALS AND METHODS

2.1 | Fish collection

Gill tissues were collected in the field from two separate sampling sites in proximate H_2S-rich (sulphidic) (PS0, Lat: 17.43843, Long: −92.77476) and nonsulphidic springs (Bonita, Lat: 17.42685, Long: −92.75213) in the Tacotalpa Drainage in southern Mexico in 2010, for the mRNA analyses, and 2015, for the miRNA analyses. All fish were collected using a seine (2 × 5 m, 3 mm mesh size), immediately euthanized using decapitation, and gills were extracted. Gill tissues were immediately placed in RNALater and transported to the field station where they were stored at 4°C until arrival at Kansas State University and then kept frozen at −20° until RNA extraction and library preparation. For the miRNA analyses, three males and three females were collected from each of the sampling sites (Table S1). For the mRNA analyses, only females were collected, $n = 6$ for the sulphidic and $n = 6$ for the nonsulphidic sites. All procedures were performed following approved Kansas State University IACUC #3473.

2.2 | RNA extraction, library preparation and sequencing

Total RNA was extracted from gill tissues with a miRNeasy mini kit (Qiagen) following the manufacturer's protocol. Total RNA was submitted to the Kansas University sequencing facility for miRNA library preparation using the Illumina TruSeq Small RNA library kit. The small RNA libraries were sequenced using an Illumina HiSeq 2500 sequencer in the Rapid Read operational mode with a 50-cycle single read (SR) sequencing strategy. HiSeq Rapid SR Cluster Kit v2 (GD-402-4002) and HiSeq Rapid SBS Kit v2 (FC-402-4022) cluster and sequencing chemistry were used to execute the run.

2.3 | Small RNA read analysis and miRNA annotation

The resulting FASTQ sequencing files were quality controlled with fastQC (version 0.11.4) (Andrews, 2010) and then trimmed with TrimGalore! (version 0.4.1) (Krueger, 2014) (*trim_galore -q 30 --three_prime_clip_R1 24 --stringency 5 --length 1*). The trimmed reads were converted to FASTA format and aligned to the *P. mexicana* reference genome (NCBI accession GCA_001443325.1, (Warren et al., 2018)) using bbmapskimmer.sh of the BBMap suite (Bushnell, et al., 2014), with parameters as in (Desvignes et al., 2019). All libraries were simultaneously analysed using Prost! (version 0.7.60) to select for read length 17–25 nucleotides. We configured Prost! to retain only sequences with a minimum of five identical reads for the annotation of miRNA genes and mature miRNAs as previously described (Desvignes, Batzel, et al., 2019) and as detailed in the Prost! documentation page (https://prost.readthedocs.io). The miRNA and isomiR nomenclature follow the rules established for zebrafish (Desvignes et al., 2015; Desvignes, Loher, et al., 2019). For expression analysis, only sequences with a minimum of 30 reads were retained. For ambiguous isomiR reads that were equally likely to be derived from multiple miRNAs, we partitioned their read counts proportionally to the expression of unambiguously annotated miRNAs that could have given rise to the isomiR. In the current study, we configured Prost! to use all mature and hairpin sequences from chordates in miRBase Release 22 (Kozomara & Griffiths-Jones, 2014), as well as the stickleback miRNA annotations (Desvignes, Batzel, et al., 2019), the Blackfin Icefish annotation (Kim, Amores, et al., 2019), the extended zebrafish miRNA annotation (Desvignes et al., 2014) and the gar miRNA annotation (Braasch et al., 2016).

2.4 | MicroRNA differential expression analysis

From the Prost! output, we used the non-normalized counts of annotated miRNA reads as input to perform differential expression analysis using the DEseq2 package (version 1.26.0) (Love et al., 2014). We used the 'parametric' type trend line fitting model (FitType) and a multi-factor design with sex and population as the factors (~ sex + population). For determining significance, we used a stringent maximum adjusted *p*-value of 1% (Benjamini and Hochberg procedure to adjust for multiple testing (Benjamini & Hochberg, 1995)) to consider miRNAs as differentially expressed between the sulphidic and nonsulphidic populations. Heat maps were generated using the Broad Institute Morpheus webserver (https://software.broadinstitute.org/morpheus/) using log2-transformed normalized counts from annotated miRNAs. Hierarchical clustering on both rows and columns was performed using the 'one minus Spearman rank correlation' model and the 'average' linkage method. To visualize variation among samples, normalized miRNA expression levels were also subjected to multidimensional scaling (MDS) using the MDSplot function from the limma package in R (Ritchie et al., 2015).

2.5 | MicroRNA target prediction

To generate a list of putative targets of the differentially expressed miRNAs, we predicted which 3′ UTRs from the *P. mexicana* gene set would be targeted by those miRNAs. The annotated 3′ UTRs for *P. mexicana* were downloaded from Ensembl (Howe et al., 2020; Yates et al., 2020) BioMart (Kinsella et al., 2011). The mature miRNA

sequence for each of the significantly differentially expressed miRNAs and the annotated UTRs were input into miRAnda (Enright et al., 2003) with parameters *-sc 140* (score threshold), *-en −20* (energy threshold), *-scale 4.0* (scaling parameter), *-go 4.0* (gap-open penalty) and *-ge 9.0* (gap-extend penalty). We removed pme-miR-737-5p because the seed is a stretch of Ts so it matches stretches of As; therefore, any UTR with a polyA would be marked as a predicted target for this miRNA.

2.6 | mRNA sequencing library preparation

For the mRNA study, RNAseq reads were previously published in (Kelley et al., 2016). Briefly, total RNA was isolated from gills with a Covaris Cryoprep and extracted using the Qiagen RNeasy Plus mini kit. PolyA+ mRNA was enriched using the Invitrogen Dynabead mRNA purification kit. mRNA was fragmented to an average size of 400 nt with the NEB mRNA Fragmentation Module. First-strand cDNA was synthesized using Invitrogen double-stranded cDNA kit, with 1 µl of a mix of random hexamers:oligo dT primers (2 µg:1 µg) for priming. The second-strand cDNA was synthesized using the NEBNext mRNA Second Strand Synthesis kit. Libraries were individually barcoded and prepared using the Kapa Library Amplification Readymix. Libraries were sequenced on an Illumina HiSeq 2000 with paired-end 101 base pair (bp) reads at the Stanford Center for Genomics and Personalized Medicine.

2.7 | mRNA read analysis

The resulting FASTQ sequencing files were quality controlled with fastQC (version 0.11.4) (Andrews, 2010) and then trimmed with TrimGalore! (version 0.4.9) (Krueger, 2014) (with Cutadapt v1.9 (Martin, 2011)). First, we adapter trimmed requiring a six bp match to the Illumina adapter with an additional hard clip at the 5' end of 11 bp. The flag *--quality 0* was used to prevent quality trimming from occurring in this step. Second, we used quality trimming to remove the 3' ends of reads that fell below a Phred score of 24 and removed reads shorter than 50 bp. We used HISAT2 (version 2.1.0) (Kim et al., 2019) to index the reference genome for *P. mexicana* (RefSeq assembly: GCF_001443325.1) (Warren et al., 2018) with the mitochondrial genome appended (GenBank: KC992995.1) (Pfenninger et al., 2014). We then used HISAT2 to map the trimmed reads to the reference genome. We used the --downstream-transcriptome-assembly (*--dta*) flag to output SAM files with XS tags for spliced alignments indicating the genomic strand the reads aligned to (+ for coding, − for template), the ideal format for StringTie. We converted the SAM files to BAM format and sorted by coordinate using SAMtools (version 1.7; Li et al., 2009). We generated mapping statistics using CollectAlignmentSummaryMetrics in Picard (version 1.141) (Institute, 2020). We used StringTie (version 2.0.3) (Pertea et al., 2015) with reference genome annotations included in Gene Feature Format 3 (GFF3) format (GCF_001443325.1 annotations) with the mitochondrial annotations appended (GenBank: KC992995.1; Pfenninger et al., 2014) to generate GTF files of assembled transcripts. The mitochondrial annotations in the GFF3 file were manually edited to replace features labelled 'tRNA' with 'gene' and gene attributes numbers replaced with higher numeric values to not overlap genes in the nuclear genome annotation file (edited version posted to GitHub). The associated Python script prepDE.py, available with StringTie (Pertea et al., 2015), was modified to print STRG identifiers after the first dictionary was created to standard output (see GitHub for details), and then used to generate a gene counts matrix. The remaining STRG identifiers were removed from the gene counts matrix.

2.8 | mRNA differential expression analysis

We used DESeq2 (version 1.26.0) (Love et al., 2014) to conduct differential gene expression analyses using the gene counts matrix from StringTie as input. We used the 'local' type trend line fitting model (FitType). For determining significance, we used negative binomial model with a stringent maximum adjusted p-value of 1% (Benjamini and Hochberg procedure to adjust for multiple testing (Benjamini & Hochberg, 1995)) to consider mRNAs as differentially expressed between the sulphidic and nonsulphidic populations. Annotations for differentially expressed genes were obtained from the annotated GFF3 file for *P. mexicana* (GenBank: GCF_001443325.1). Ensembl gene stable IDs and their associated NCBI gene (formerly Entrezgene) accessions were downloaded from Ensembl BioMart (Kinsella et al., 2011) (Ensembl Genes 100 Dataset, Shortfin molly genes [P_mexicana-1.0]) and intersected with the genes list. All significantly differentially expressed genes without matching Ensemble gene stable IDs were hand annotated, and missing IDs were added. We visualized variation in mRNA expression using the MDSplot function from the limma package in R (Ritchie et al., 2015).

2.9 | Selection of differentially expressed targets

Once the lists of differentially expressed genes and differentially expressed miRNAs were obtained, we intersected the two lists to identify all significantly differentially expressed miRNAs that display an inverse expression pattern with significantly differentially expressed predicted target genes. Given the repressive function of miRNAs, if the miRNA was over-expressed, its targets should be downregulated, and if the miRNA is downregulated, its targets should be upregulated. The predicted mRNA targets that have expression inverse to miRNA expression were then input into GOrilla (Eden et al., 2009) to identify which pathways were significantly altered due to a significant change in miRNA expression. The reference set included the 16,740 genes that have annotated 3′ UTRs in the *P. mexicana* reference genome. The vast majority had SwissProt hits in the human database (16,014), which matched to 9,017 genes, 8,903 of which were associated with

a GO term. We considered GO terms to be enriched if the corrected *p*-value (Benjamini & Hochberg, 1995) was less than 5%.

3 | RESULTS

3.1 | miRNA genes and mature miRNAs annotations in *P. mexicana*

Lacking a comprehensive miRNA annotation for *P. mexicana* (Fromm et al., 2019; Kozomara & Griffiths-Jones, 2014), we annotated miRNA genes and mature miRNAs using the small RNA sequencing data produced in this study and aligning them to the *P. mexicana* reference genome (GCA_001443325.1) using Prost! (Desvignes, Batzel, et al., 2019). The analysis recovered a total of 320 individual mature miRNAs originating from 291 miRNA genes among which 127 are organized in 52 clusters (Table S2, Appendix S1 and Appendix S2). Among the miRNA genes, 163 (i.e. 56.0%) have both 5p and 3p strands annotated, 59 (i.e. 20.3%) have only the 5p strand annotated, and 44 (i.e. 15.1%) have only the 3p strand annotated. An additional 25 genes (i.e. 8.6%) corresponding to orthologs of known teleost miRNAs were annotated after synteny verification although no reads for these genes were present in our sequencing data.

Of the mature miRNAs that we identified in the *P. mexicana* genome, 318 had a total read count above 30 and were included in the differential expression analysis (Table S3, Figure S1). The samples separated by population of origin (sulphidic versus nonsulphidic) along the first axis of the MDS (Figure 1). Only the nonsulphidic population separated by sex on the second axis of the MDS. A total of 90 miRNAs were differentially expressed between sulphidic and nonsulphidic samples with 46 miRNAs significantly upregulated in sulphidic conditions and 44 miRNAs significantly downregulated (FDR < 0.01) (Figure 2). Two miRNAs (pme-miR-8160a-5p and pme-let-7g-5p) were downregulated in females compared to males (FDR < 0.01).

There were 17,534 transcripts, corresponding to 12,991 unique genes, with annotated 3′ UTRs in Ensembl. Each of the significantly differentially expressed miRNAs had on average 413 putative targets in the 3′ UTRs (range 43 to 1,663 targets), with a total of 36,574 predicted targets, corresponding to 9,708 unique genes.

3.2 | mRNA read analysis

An analysis of the mRNA data set from (Kelley et al., 2016; for a detailed discussion see Kelley et al., 2016) revealed that sulphidic individuals clustered separately from nonsulphidic individuals in an MDS (Figure 1b). Of the 21,251 genes with sufficient data to be included in the analysis, a total of 2,132 genes were significantly differentially expressed in the sulphidic population compared to the nonsulphidic population (FDR < 0.01; Table S4). Of the significantly differentially expressed genes, 1,660 had Ensembl IDs (1,633 in the database and 27 hand annotated).

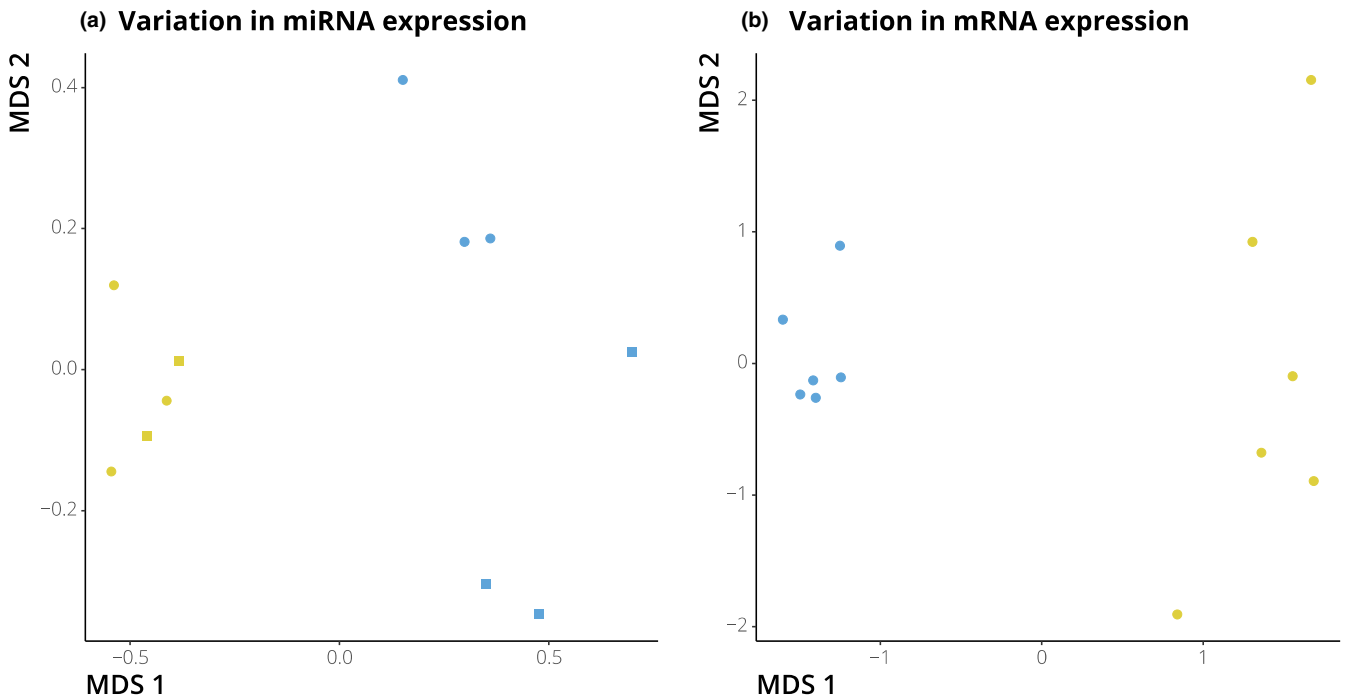

FIGURE 1 (a) Multidimensional scaling (MDS) plot of miRNA gene expression profiles among samples from nonsulphidic (NS) (blue) and sulphidic (S) (yellow) habitats, calculated from all miRNAs. Males (squares) and females (circles) are also indicated. (b) MDS of distances between mRNA gene expression profiles among samples from nonsulphidic (NS) (blue) and sulphidic (S) (yellow) habitats, calculated from the top rlog transformed counts for all mRNAs

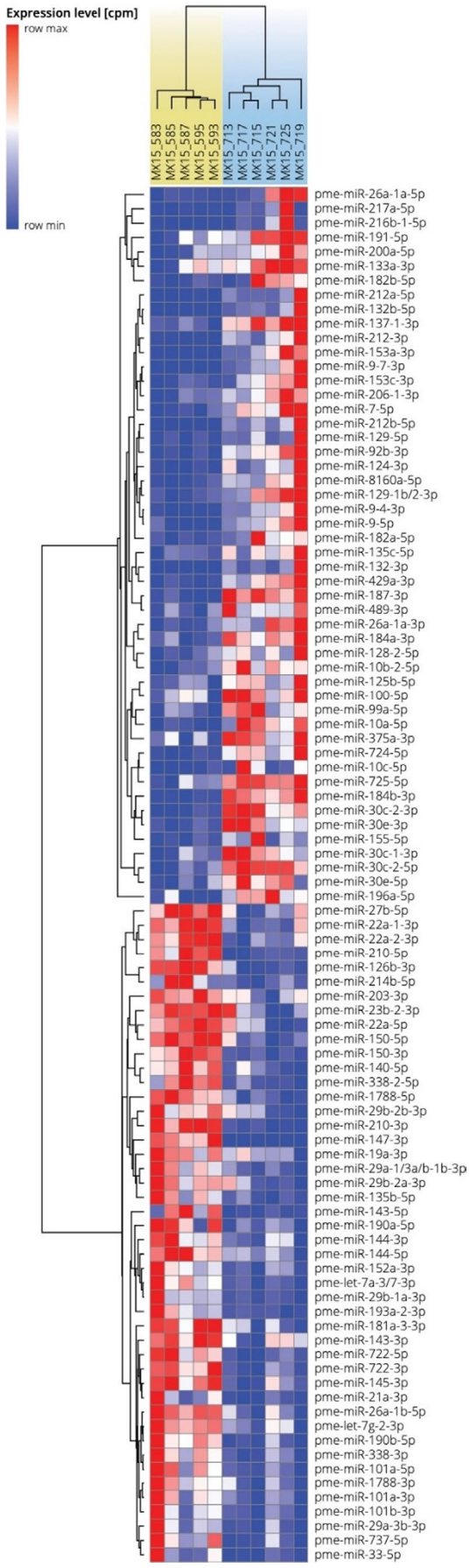

FIGURE 2 Heat map of the normalized expression data of the 90 *P. mexicana* mature miRNAs that were differentially expressed between sulphidic (yellow) and nonsulphidic (blue) populations

3.3 | mRNA targets potentially regulated by miRNAs

To determine which protein-coding genes and pathways were most likely significantly regulated by miRNAs, we intersected the differentially expressed miRNAs with the differentially expressed mRNAs and analysed the retained data set with GOrilla. We first sub-selected for the differentially expressed mRNAs that were predicted targets of the differentially expressed miRNAs. And second, given the action of miRNAs, we retained only the pairs of miRNAs and mRNAs with inverse expression patterns. Forty-six miRNAs that were significantly upregulated in sulphidic individuals targeted 320 unique genes (294 with Swiss-Prot accessions), for a total of 718 miRNA-mRNA pairs with inverse expression patterns. The predicted mRNA targets that were significantly downregulated in the sulphidic environment were significantly enriched for the mitochondrion component (GO:0005739; FDR = 0.0424). The topmost significant biological process GO terms were small molecule metabolic process (GO:0044281; FDR = 8.95E-04), chemical homeostasis (GO:0048878; FDR = 7.44E-04) and organic acid metabolic process (GO:0006082; FDR = 1.24E-03) (Table S5). The 44 miRNAs that were significantly downregulated in sulphidic individuals targeted 470 unique genes (421 with Swiss-Prot accessions), for a total of 1,101 inversely correlated miRNA-mRNA pairs. The predicted mRNA targets that were upregulated in the sulphidic environment were enriched for the plasma membrane component (GO:0005886; FDR = 2.06E-04), extracellular region part component (GO:0044421; FDR = 5.31E-04) and plasma membrane part component (GO:0044459; FDR = 3.86E-03). The topmost significant biological processes GO terms upregulated in the sulphidic environment were responses to stimuli, including but not limited to, response to endogenous stimulus (GO:0009719; FDR = 5.64E-05), response to chemical (GO:0042221; FDR = 8.03E-05) and negative regulation of multicellular organismal process (GO:0051241; FDR = 1.49E-04) (Table S5).

Whereas not the most significant GO term in the Biological Process GO ontology, hydrogen sulphide metabolic process (GO:0070813) was significantly enriched in the set of targets upregulated in the sulphidic environment (FDR = 0.00769). Four of five genes in that GO term were differentially expressed including *sqrdl* (sulphide quinone reductase), *cbs* (cystathionine-beta-synthase), *mpst* (mercaptopyruvate sulfurtransferase) and *ethe1* (ethylmalonic encephalopathy 1) (Figure 3).

4 | DISCUSSION

We characterized, for the first time, miRNAs in *Poecilia mexicana*, a species whose ability to successfully colonize and adapt to extreme environments is in part driven by the evolution of gene regulation

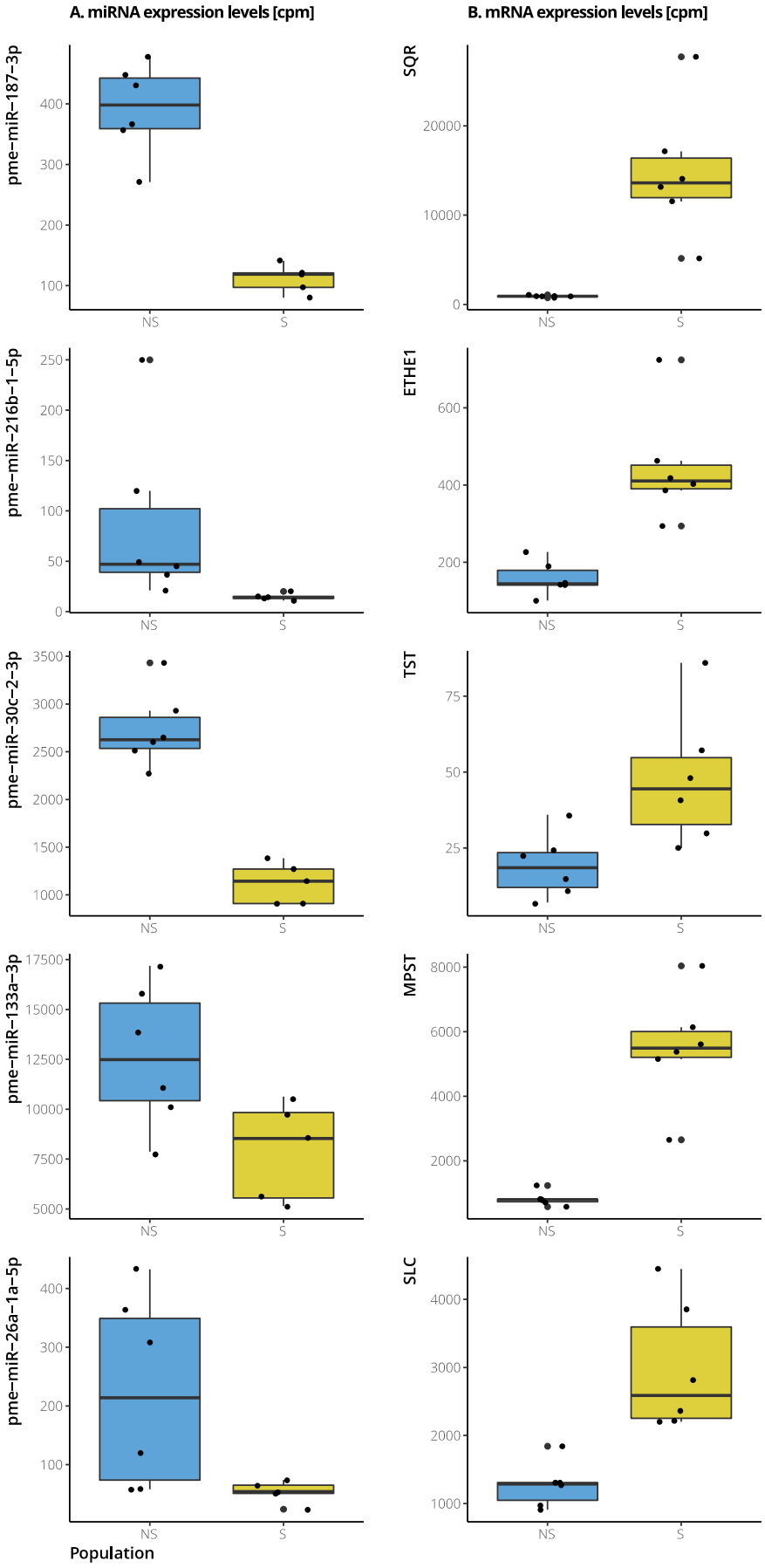

FIGURE 3 (a) Expression of specific miRNAs and a selection of the (b) candidate genes that the miRNAs were predicted to target that are associated H_2S detoxification, separated by nonsulphidic (NS, blue) and sulphidic (S, yellow) populations. All miRNAs and mRNAs shown were significantly differentially expressed based on the DEseq2 analysis in R, FDR < 0.01

(Tobler et al., 2018). Overall, we detected and annotated 320 mature miRNAs that were produced by 291 miRNA genes. A significant portion of these miRNAs was differentially expressed between populations locally adapted to contrasting environmental conditions, suggesting that miRNAs could be involved in the adaptive regulation of gene expression. Comparing miRNA expression to existing transcriptome data, we were able to specifically identify miRNAs whose expressions were inversely correlated with the expression of target mRNAs. Consistent with selection stemming from toxic H_2S in our study populations, we found that some differentially expressed miRNAs appear to regulate the expression of genes associated with H_2S detoxification. Overall, our results suggest that miRNAs provide a promising new avenue to explore the regulatory mechanisms involved in local adaptation to H_2S and the convergent evolution of gene expression.

Our new annotation of miRNAs also provides an important addition to the growing genomic resources for *P. mexicana*, which include a variety of transcriptome data sets (Kelley et al., 2012) and a draft reference genome that lacked miRNA annotations (Warren et al., 2018). Improving these resources has the potential to impact future research, since *P. mexicana* is not only studied in the context of adaptation and speciation in sulphide springs (Tobler et al., 2018), but also in the context of animal behaviour (Bierbach et al., 2013; MacLaren & Rowland, 2006; Marler & Ryan, 1997; Plath et al., 2004), predator–prey interactions (Bierbach et al., 2011; Tobler et al., 2007), sensory ecology (McGowan et al., 2019; Tobler et al., 2010), life history (Riesch et al., 2009, 2010) and the origin of unisexual species (Schartl et al., 1995; Schlupp, 2005). Comparing *P. mexicana* miRNAs to those of other teleost fishes, we found a number of orthologs across species. The number of miRNA genes in *P. mexicana* is comparable to that in other species, which range between 275 and 308 (Desvignes, Batzel, et al., 2019; Kim, Amores, et al., 2019). However, the number of annotated mature miRNA is lower in *P. mexicana*, as other teleosts exhibit between 408 and 495 mature miRNAs. The sequencing of a more diverse array of organs and developmental stages from *P. mexicana* would likely improve the annotation of mature miRNAs. In this study, we primarily focused on the gills, because these play a critical role in adaptation to environmental H_2S (Passow, Brown, et al., 2017).

Most importantly, we found significant differential expression of miRNAs between adjacent populations that live in either a H_2S-rich spring or a nonsulphidic stream. A wide variety of studies have focused on these populations and documented adaptive trait variation, ranging from analyses of divergent genotypes, molecular phenotypes associated with gene expression and enzyme function, to emergent phenotypes associated with physiology, morphology, behaviour and life history (Tobler et al., 2018). Differentially expressed miRNAs therefore have the potential to play a role in the expression of adaptive trait differentiation between the sulphidic and nonsulphidic populations. Although originating from different individuals from the same populations, the prediction of mRNA targets of differentially expressed miRNAs uncovered inverse expression patterns between miRNAs and a variety of mRNAs that have previously been linked to adaptation to H_2S. Most importantly, this pertains to genes associated with H_2S detoxification (Figure 3), which includes multiple genes of the SQR pathway and a sulphate transporter that are all significantly upregulated in sulphide spring populations (Kelley et al., 2016). The downregulation of miRNAs that we documented in the sulphidic population may increase the stability and longevity of target mRNAs, leading to a higher net expression of detoxification genes in the sulphidic population.

Among predicted mRNA targets that had reduced expression in the sulphidic population (with targeting miRNAs upregulated), 16.6% were predicted to localize to the mitochondria based on GO enrichment, the only significantly enriched cellular component identified by the GO term enrichment analysis. The mitochondria are the primary site of H_2S toxicity and detoxification; thus, miRNAs may help tune down mitochondrial functions and protect from H_2S toxicity. The upregulation of miRNAs may accelerate the breakdown of mRNAs associated with succinate dehydrogenase (Complex II in the ETC), leading to a predicted downregulation of the genes when mitochondria operate under high H_2S concentrations (Kelley et al., 2016). Among upregulated miRNAs, miR-133a-3p, which was differentially expressed between the sulphidic and nonsulphidic populations, is responsive to H_2S exposure in human cardiomyocytes (Ren et al., 2019). This potentially indicates that H_2S-dependent differential expression of certain miRNAs has been highly conserved throughout the diversification of animals, just like H_2S-toxicity and detoxification mechanisms (Shahak & Hauska, 2008; Tobler et al., 2016).

The negative correlations between the expression of miRNAs and mRNAs previously implicated in H_2S adaptation opens a number of important questions to explore the role of miRNA in adaptive evolution. (a) Future studies need to test whether miRNA expression patterns are the consequence of plasticity shaped by population-specific exposure histories, or whether there is heritable variation between populations. Past research has shown that a large number of genes that are differentially expressed between sulphidic and nonsulphidic populations in nature remain differentially expressed in the laboratory or exhibit evolved, population-specific responses to H_2S exposure (Passow, Henpita, et al., 2017). Thus, combined common-garden and H_2S-exposure experiments will be needed to disentangle the effects of heritability and plasticity on miRNA expression in this system. (b) Additional work will also need to validate the in vivo interactions between miRNAs and their predicted targets, because the regulatory function of miRNAs can differ greatly based on target abundance and interaction site features leading to effects ranging from significant to undetectable at the protein level (Denzler et al., 2016; Fridrich et al., 2019; McGeary et al., 2019). Our in silico approach identified a large number of putative mRNA targets for differentially expressed miRNAs, and it is unlikely that all of these targets will have functional ramifications on in vivo mRNA levels and ultimately organismal function. (c). Future research will also need to characterize the causal effects of specific miRNAs on their targets to test whether reducing the levels

of miRNAs that have predicted targets related to H_2S detoxification genes will increase H_2S detoxification capability and tolerance in cells from nonsulphidic fish. (d) Last but not least, a core finding of past research has been that different lineages of sulphide spring fishes exhibit strong patterns of convergent evolution in gene expression. This is true for different lineages within the *P. mexicana* species group that have independently colonized H_2S-rich springs in southern Mexico (Kelley et al., 2016), as well as for species from other poecilid genera that span over 40 million years of evolution (Greenway et al., 2020). Thus, a major remaining question is whether the evolution of miRNA expression ultimately underlies the convergent evolution of gene expression evident across these lineages. Addressing that question will require the characterization of miRNAs and their expression patterns among the different lineages that have colonized sulphide springs as well as ancestral lineages that are present in nonsulphidic freshwater environments.

In summary, we have annotated miRNAs in the *P. mexicana* genome and demonstrated that a significant portion of miRNAs were differentially expressed between locally adapted populations. This study opens up the possibility that miRNAs could play an important role during adaptive evolution and the colonization of extreme environments. miRNAs can buffer variation in gene expression under stressful conditions (Leung & Sharp, 2010) and modifications of miRNAs or their targets could accelerate the adaptive process. miRNAs can also spur speciation through new physiological capabilities and/or the generation of new morphotypes (Arif et al., 2013). Indeed, miRNAs have been hypothesized to play a role in species divergence (Loh et al., 2011). Given the multiple regulatory mechanisms modulating gene expression, future efforts need to especially focus on studying integrated regulatory processes to disentangle the regulatory mechanisms contributing to adaptive evolution.

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AUTHOR CONTRIBUTION

M.T., Z.C., J.L.K. and L.A.R. conducted fieldwork. T.D., K.L.M., J.L.K., M.P., A.P.B. and Z.C. conducted analyses. M.T. contributed to the statistical design. T.D., K.L.M., M.T. and J.L.K. wrote the manuscript; all authors contributed to revisions. All authors approved the final version.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

All microRNA sequence data are available in NCBI BioProject PRJNA471100. All RNAseq data are available in NCBI BioProject PRJNA290391. Code is available at github.com/jokelley/pmex-wild-microRNA.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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