Physiol Genomics 49: 505–518, 2017.
First published August 11, 2017; doi:10.1152/physiolgenomics.00016.2017.

RESEARCH ARTICLE | *General Interest*

Small noncoding RNA expression during extreme anoxia tolerance of annual killifish (*Austrofundulus limnaeus*) embryos

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Submitted 15 February 2017; accepted in final form 9 August 2017

Riggs CL, Podrabsky JE. Small noncoding RNA expression during extreme anoxia tolerance of annual killifish (*Austrofundulus limnaeus*) embryos. *Physiol Genomics* 49: 505–518, 2017. First published August 11, 2017; doi:10.1152/physiolgenomics.00016.2017.—Small noncoding RNAs (sncRNA) have recently emerged as specific and rapid regulators of gene expression, involved in a myriad of cellular and organismal processes. MicroRNAs, a class of sncRNAs, are differentially expressed in diverse taxa in response to environmental stress, including anoxia. In most vertebrates, a brief period of oxygen deprivation results in severe tissue damage or death. Studies on sncRNA and anoxia have focused on these anoxia-sensitive species. Studying sncRNAs in anoxia-tolerant organisms may provide insight into adaptive mechanisms supporting anoxia tolerance. Embryos of the annual killifish *Austrofundulus limnaeus* are the most anoxia-tolerant vertebrates known, surviving over 100 days at their peak tolerance at 25°C. Their anoxia tolerance and physiology vary over development, such that both anoxia-tolerant and anoxia-sensitive phenotypes comprise the species. This allows for a robust comparison to identify sncRNAs essential to anoxia-tolerance. For this study, RNA sequencing was used to identify and quantify expression of sncRNAs in four embryonic stages of *A. limnaeus* in response to an exposure to anoxia and subsequent aerobic recovery. Unique stage-specific patterns of expression were identified that correlate with anoxia tolerance. In addition, embryos of *A. limnaeus* appear to constitutively express stress-responsive miRNAs. Most differentially expressed sncRNAs were expressed at higher levels during recovery. Many novel groups of sncRNAs with expression profiles suggesting a key role in anoxia tolerance were identified, including sncRNAs derived from mitochondrial tRNAs. This global analysis has revealed groups of candidate sncRNAs that we hypothesize support anoxia tolerance.

miRNA; mitochondria; diapause; preconditioning

MOST VERTEBRATE SPECIES experience tissue damage or die if denied oxygen for even a brief period of time, yet a few remarkable species can survive for weeks to months in the complete absence of oxygen (anoxia). Embryos of the annual killifish *Austrofundulus limnaeus* are the most anoxia-tolerant vertebrate species known, surpassing even the impressive anoxia tolerance of the crucian carp and western painted turtle (82). The majority of anoxia-tolerant species, including *A. limnaeus*, quickly and profoundly depress their metabolism when faced with anoxia, while intolerant organisms exhibit physiological changes but do not enter as profound of a state of hypometabolism (72). Surviving anoxia requires the organism to successfully enter, sustain, and emerge from metabolic depression with the appropriate timing. Global suppression of protein synthesis characterizes metabolic depression, though upregulation of specific proteins is necessary to support the hypometabolic state (14, 67). Precise changes in gene expression are likely necessary to support the required changes in cell and organismal physiology associated with anoxia-induced quiescence.

Small noncoding RNAs (sncRNA), such as microRNAs (miRNAs), can specifically, rapidly, and reversibly modulate gene expression (10). MiRNAs play roles in the regulation of most physiological processes, including development (21, 96, 101), responses to ischemia (4, 18, 28, 54), and the regulation of metabolism (53) and have recently emerged as potentially important players in mediating events associated with diverse examples of metabolic depression and environmental stress. This suite of characteristics and expression patterns makes sncRNAs a compelling focus to understand the mechanisms of long-term anoxia tolerance. By conducting a comparative study of sncRNA expression in different stages of *A. limnaeus* embryos that exhibit variable anoxia tolerance and physiology over development we identify sncRNAs that we hypothesize support vertebrate anoxia tolerance.

MiRNAs, the most thoroughly studied class of sncRNAs, dynamically and specifically modulate gene expression. They are involved in a myriad of cellular processes from metabolism (53) and cell cycle regulation (20) to developmental signaling (43, 93). Genes for canonical miRNAs are encoded by the nuclear genome and transcribed, but not translated. Transcripts are processed into mature miRNAs (9) that bind to complementary nucleotide sequences of target mRNAs (9), ultimately resulting in either degradation or reduced translation of the mRNA (1, 9, 10, 49). A single miRNA may target many mRNA transcripts, thereby affecting a suite of genes and fundamentally altering gene expression and the physiological state of the organism (9, 40).

MicroRNAs are predicted to regulate over half of the human mRNA transcriptome, and predictions indicate that selective pressure has maintained complementary miRNA-mRNA binding (34). Thus, miRNAs are an essential component of post-transcriptional gene regulation. Recent analysis also points to an interplay between miRNAs and other forms of post-transcriptional gene regulation, regulating gene expression networks in concert (41). Rapid miRNA turnover, ranging from less than an hour to several hours (86), and rapid mRNA deadenylation signaling for degradation by miRNAs (9, 107) allow for quick regulation of mRNA stability and translation. Additionally, miRNAs are found and highly conserved throughout the animal kingdom (9).

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Entry into metabolic depression, in response to environmental stress or genetic programming, induces differential expression of miRNAs. Hibernating bats (16, 109) and ground squirrels (58, 106) and aestivating sea cucumbers (22, 23) differentially express miRNAs when entering their respective forms of hypometabolism. Frogs exposed to freezing (7, 12) and dehydration (105, 106), turtles exposed to anoxia (95), as well as invertebrates exposed to anoxia (13) and freezing (65, 66) all differentially express miRNAs in response to hypometabolism induced by the environment. These hypometabolism-responsive miRNA expression patterns vary among and within organisms, by tissue type and treatment.

Stress also induces changes in miRNA expression, even in organisms that do not enter metabolic depression. Many studies on cell lines of anoxia-sensitive species (human and mouse) reveal hypoxia-regulated miRNAs (HRM) and hypoxia-associated miRNAs (hypoxamirs) (54–56). Many HRMs target proteins involved in metabolism, ranging from glycolytic pathways and function of the citric acid cycle, to apoptosis (27). Several hypoxamirs expressed in the heart are involved in mitochondrial physiology (biogenesis, respiration, fission, and abnormalities), as well as cardiomyocyte apoptosis (4). The majority of HRMs and hypoxamirs increase in abundance in response to anoxia. Furthermore, ischemic preconditioning (nonlethal occlusion of blood flow that increases tolerance to future ischemic events) alters miRNA expression in mice (60), indicating that miRNA expression changes may confer important physiological shifts necessary to extend survival of anoxia, not just to immediately respond to the lack of oxygen.

While most studies analyzing sncRNA expression in response to hypoxia and hypometabolism focus on miRNAs, other classes of sncRNAs are also emerging as important players in the regulation of cellular and organismal function. SncRNAs are also derived from tRNA, rRNA, long noncoding RNA, small nucleolar RNA, and piwi-RNA; many of these have gene regulatory functions (32, 35, 36, 51, 85, 99, 100). Environmental stressors, such as dehydration and oxidative stress, generate tRNA-derived fragments in diverse organisms, ranging from yeast to humans (36, 37, 47, 61, 87, 88). Classes of mitochondria-associated sncRNAs have also come into view. A new class of miRNAs, called mitomiRs, localize to the mitochondria, either by translocation or physical association (5, 6, 8, 59, 94). The mitochondrial genome also produces sncRNAs, termed mitosRNAs (84). Many sncRNAs have miRNA-like gene regulatory properties (32); however, discovery of new functions and mechanisms continues. Recent advances in understanding the roles of miRNA in modulation of gene expression, particularly during metabolic depression and anoxia, hypoxia, or ischemia, make sncRNAs a promising avenue of investigation of anoxia tolerance mechanisms in *A. limnaeus* embryos.

The ephemeral pond habitat of *A. limnaeus* imposes extreme environmental conditions, including anoxia, on developing embryos (50, 79). As a result, extreme anoxia tolerance is critical to the persistence of the species. Three periods of metabolic dormancy, called diapause, lengthen embryonic development and help the embryos endure the extreme environment of the dry season (76, 80, 82). Different developmental stages of *A. limnaeus*, with unique physiology and morphology, display different levels of anoxia tolerance (Fig. 1). Tolerance peaks during diapause 2 (D2), when embryos survive for 90–120 days without oxygen (80). During early post-D2 development, metabolically active embryos maintain this extreme tolerance of anoxia for at least 4 days post-D2 (dpd). These embryos enter an anoxia-induced quiescence characterized by an arrest of development, cessation of cardiac activity, and severe metabolic depression (81). After 4 dpd, as embryos approach hatching, their anoxia tolerance gradually declines (80, 82). By 12 dpd embryos survive about 1 wk without oxygen (80, 82) and by 20 dpd, shortly before hatching, embryos survive less than 24 h of anoxia (Fig. 1). Additionally, anoxic preconditioning (82), a brief nonlethal exposure to anoxia, extends the anoxia tolerance of 8–12 dpd stage embryos, as previously described in mammals. Precondition-

WS[1]	dpd[2]	LT_{50}, days[3]	AP[4]	Sampling (hrs)[5]	Metabolic Depression[6]	Heart Rate[7]	Morphology	
32	0	62	Unknown	0, A4, A24 R2, R24	Diapause, Quiescence	15 ± 5	Muscle segments, early tubular heart, early brain development	
36	4	74.3	-20%	0, A4, A24 R2, R24	Quiescence	78 ± 5	Increase in brain size, appearance of pigmentation, vitelline circulation	
40	12	6.7	+32%	0, A4, A24 R2, R24	Quiescence	93 ± 6	Advanced cardiovasculuar system, liver and gut present, eyes reflective	
42	20	0.66	-21%	0, A2, A6 R2, R24	Quiescence	89 ± 7	Fully formed organs, nearly ready to hatch	

Fig. 1. Anoxia tolerance, physiology, and sampling of *Austrofundulus limnaeus* developmental stages included in this study [1]WS , Wourms' stage (102). [2]dpd, Days postdiapause 2. [3]LT_{50}, time to 50% mortality in anoxia at 25°C for 0, 4, and 12 dpd embryos (80, 82). Data for 20 dpd embryos are presented here first. [4]AP, percent change in survival following anoxic preconditioning (82). Data for 20 dpd embryos are presented here first. [5]A, anoxia; R, recovery. [6]Type of metabolic depression involved. [7]Heart rate in beats/min during normoxic incubation at 25°C (3).

ing, however, does not extend the anoxia tolerance of D2 or 4 dpd embryos, the most anoxia-tolerant stages, or 20 dpd embryos, the least anoxia-tolerant stage.

A. limnaeus presents an opportunity for intraspecific comparison between embryonic stages that differ in their metabolic physiology, anoxia tolerance, and response to preconditioning. This unique model provides a framework to identify adaptive sncRNAs, essential to survival of anoxia. This study characterizes sncRNA expression during development and in response to anoxia followed by aerobic recovery in embryos that differ dramatically in their anoxia tolerance. Each stage displays a unique profile of sncRNAs under normoxic conditions, which respond distinctly to exposure to anoxia and recovery. Dormant D2 embryos display a unique sncRNA profile under normoxia and in response to anoxia, relative to the actively developing and metabolically active post-D2 embryos whose profiles are more similar to each other. Known stress- and hypoxia-responsive miRNAs are identified in the *A. limnaeus* data set. Additionally, many novel sncRNAs are also identified, including highly anoxia-responsive sequences derived from the mitochondrial genome.

METHODS

Embryo rearing and staging. Embryos of *A. limnaeus* were collected from adult spawning pairs and cared for according to established husbandry methods (75) in accordance with approved Portland State University (PSU) Institutional Animal Care and Use Committee (IACUC) protocols (PSU IACUC protocol #33). At 25°C embryos normally enter D2 at 24 days postfertilization (dpf). To study post-D2 stages, embryos were exposed to continuous light for 48 h at 30°C to break diapause. Embryos from multiple spawning events were pooled when breaking D2 to account for any spawn-specific variation. Following confirmation that embryos had broken diapause as assessed by morphology and heart rate as previously described (67), embryos were returned to 25°C where they were maintained by established methods (75). Embryos were staged according to Wourms' staging (102–104).

Experimental design. Details of the experimental design and the physiological phenotypes of the developmental stages used in this study are provided in Fig. 1. Embryos were exposed to anoxia and aerobic recovery following anoxia and sampled for sncRNA expression profiling. D2, 4 dpd, and 12 dpd embryos were sampled at 0, 4, and 24 h of anoxia and 2 and 24 h of aerobic recovery. Since 20 dpd embryos have a time to 50% mortality (LT_{50}) in anoxia of less than 24 h, their sampling was adjusted to 2 and 6 h of anoxia to fall within their tolerance. We sampled 20 dpd recovery embryos at 2 and 24 h of recovery. For each stage, four biological replicates ($n = 4$), comprising 20 embryos each, were sampled at each designated time.

Anoxic exposure and aerobic recovery. Embryos were exposed to anoxia at 25°C in a Bactron III anaerobic chamber (Sheldon Manufacturing, Cornelius, OR) as previously described (68). Following anoxia, embryos were removed from the anaerobic chamber and rinsed three times in normoxic embryo medium to initiate aerobic recovery. Embryos were allowed to recover at 25°C until sampling.

Sampling embryos for sncRNA sequencing. At the time of sampling, embryos were blotted dry on a 100 μm nylon mesh screen placed on a paper towel to wick away excess medium. Embryos were then transferred to a preweighed microcentrifuge tube and flash-frozen in liquid nitrogen. Frozen samples were stored at −80°C until RNA extraction. For anoxic samples this procedure was performed within the anaerobic chamber, and closed microcentrifuge tubes were immediately flash-frozen upon removal from the anaerobic environment.

RNA extraction. Total RNA was extracted from each sample with TRIzol reagent (Invitrogen, Carlsbad, CA), as previously described (26, 98). Briefly, samples were homogenized in TRIzol and phase-separated with chloroform, and RNA was precipitated from the aqueous phase by overnight incubation in a high-salt solution according to the manufacturer's instructions. Total RNA was resuspended in 1 mM sodium citrate (pH = 6.4). Total RNA concentration and purity were assessed by measuring absorbance at 260 and 280 nm and calculating the A_{260}/A_{280} ratios using an Infinite Pro M200 plate reader equipped with a NanoQuant plate (Tecan, San Jose, CA). To assess RNA integrity, RNA was run on a 2% agarose gel, stained with ethidium bromide and visually inspected for distinct bands representing 18S and 28S rRNA subunits. The mean A_{260}/A_{280} ratio was 2.15 ± 0.05 (Supplemental Table S1). (The online version of this article contains supplemental material.) Only high-quality RNA samples with A_{260}/A_{280} ratios ranging from 1.92 to 2.2 and distinct bands for 18S and 28S rRNA ribosomal subunits were used to prepare sequencing libraries. Total RNA was stored at −80°C until cDNA library preparation.

sncRNA sample preparation. cDNA libraries were prepared from 1 μg total RNA as input for the TruSeq Small RNA sequencing kit (Illumina, San Diego, CA), following the manufacturer's guidelines. Adapter-ligated small RNAs were reverse-transcribed and amplified by PCR. Due to low small RNA abundance in our embryos, particularly at younger stages, double the recommended amount of ligated RNA was used for PCR amplification. cDNA libraries were purified by gel electrophoresis on a 6% polyacrylamide gel and small RNAs, determined by bands corresponding with 22–30 nucleotide long fragments, were excised from the gel. cDNA was purified by ethanol precipitation and resuspended in 10 mM Tris·HCl, pH 8.5. Samples were stored at −20° C until library validation. Sequencing library quantity, quality, and sequencing were performed at the Oregon Health and Sciences University Massively Parallel Sequencing Shared Resource. Library quality was assessed before sequencing using a DNA-1000 chip on a model 2100 Bioanalyzer (Agilent Technologies). Real-time quantitative PCR was used to quantify libraries before cluster generation and sequencing on an Illumina HiSeq 2000. Single end sequencing was run for 36, 50, or 100 cycles, depending on the other samples being run at the time (Supplemental Table S1). All cycle lengths captured the small RNAs and part of the 3′-adapter sequence. Twelve samples were multiplexed per flow cell lane. Unique indexes tagged to adapter sequences were used to computationally demultiplex reads after sequencing. Biological replicates were distributed across flow cell lanes to eliminate lane-bias. Bcl2fastq2 version 2.1.17.1.14 was used to initially process the data and generate fasta files for analysis. Raw sequence files have been deposited in the NCBI sequence read archive. Accession numbers are listed in Supplemental Table S1.

sncRNA sequence processing and analysis. Samples were processed according to the following pipeline. Reads were trimmed with Trimmomatic (version 0.36) to remove 3′- and 5′-adapters and low-quality reads with a Phred score under 33 (17). Reads 15–27 nucleotides long were retained for analysis to capture canonical miRNAs as well as other known and novel classes of sncRNAs. FastQC (version 0.11.5) analysis was run and compared before and following trimming. Sequences were mapped to the *A. limnaeus* genome [Austrofundulus_limnaeus-1.0 GCF_001266775.1 and the *A. limnaeus* mitochondrial genome (97)]. Sequences with exact matches to the genome were considered real *A. limnaeus* sequences and retained for analysis. All 80 samples were normalized by library size (62), to allow for comparison of expression values. Sequences with very low expression were removed from the data by filtering to retain only sequences with a sum of normalized counts across all 80 samples > 4. Sequences in this resulting catalog (725,773 sequences) were annotated to known sncRNAs documented in miRbase v.19 (39), RFAM (version 12.1) (71), and the *A. limnaeus* mitochondrial genome (97). Annotations were conducted in CLC Genomics Workbench v6 (https://www.qiagenbioinformatics.com/), allowing for up to two mismatches and

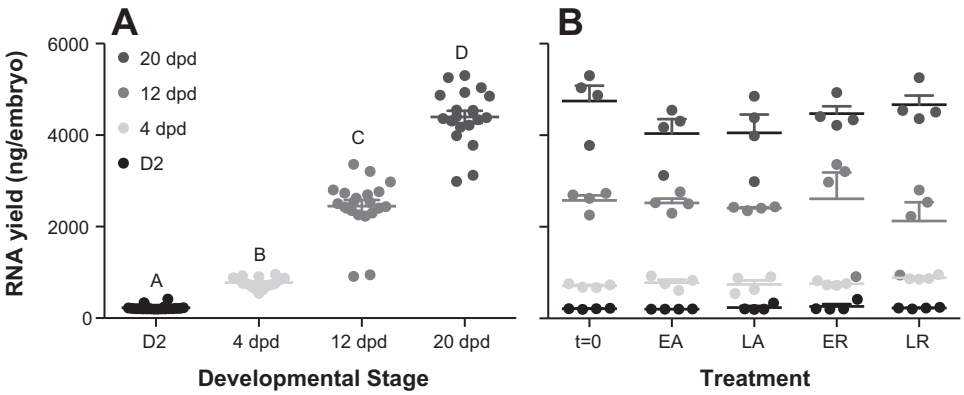

Fig. 2. RNA yield per embryo in the 4 developmental stages investigated. *A*: the total RNA extracted per embryo increases during development, likely due to cell proliferation and differentiation (ANOVA, $P < 0.0001$; Tukey's post hoc, $P < 0.05$; stages with different letters are statistically different). *B*: anoxia treatment does not alter the amount of extractable RNA (ANOVA, $P = 0.9967$). D2 , diapause 2; dpd, days postdiapause 2; $t = 0$, normoxic embryos; EA, early anoxia; LA, late anoxia; ER, early recovery; LR, late recovery. For details on the experimental treatments, see Fig. 1.

two additional or missing bases up or downstream of the annotating sequence.

Description and analysis of differential expression of the *A. limnaeus* sncRNA sequence catalog were performed using the Bioconductor package in R version 3.2.1 (38, 83). Differential expression analysis was performed across experimental treatments within each embryonic stage, as well as across all stages under normoxic conditions ($t = 0$) using DESeq2 (62). *P* values were generated by the likelihood ratio test with independent filtering to remove remaining low count sequences (2). Highly differentially expressed sequences were selected based on the following criteria: padj < 0.01; $\log_2$ fold-change > 2 or < -2; base mean > 25 normalized counts across all samples. Between stages, fold-change was calculated relative to D2 expression values for analysis of differential expression over development. Fold-change was calculated relative to $t = 0$ expression values for analysis of differential expression in response to anoxia and recovery from anoxia within a stage.

Highly differentially expressed sncRNAs of interest were clustered by expression pattern with Cluster 3.0 (31) for heat map generation. Data were log transformed and then organized by genes into clusters, based on k-means, using the Euclidean distance similarity metric for 100 runs. Clustered genes were viewed as a heat map using Java TreeView (89).

Literature search for miRNAs. To compare miRNAs identified in *A. limnaeus* to known stress, hypometabolism, ischemia, hypoxia, and ischemic preconditioning-responsive miRNAs in the literature we created a database of miRNAs known to respond to these conditions (Supplemental Table S2). The list of stress and hypometabolism-responsive miRNAs included all examples of miRNAs differentially expressed in response to hypometabolism found by searching "miRNA hypometabolism" in Google Scholar. Articles directly pertaining to hypoxia (i.e., in the title) were skipped. For the hypoxia-responsive miRNA list, we referenced the latest most comprehensive review on the topic by Kulshreshtha et al. 2008 (54) and supplemented it with a specific recent review on miRNAs in myocardial infarction (18), reviews on hypoxamirs (4, 27), and recent reviews and primary articles on miRNAs in preconditioning (28, 91, 108). MiRNA names were reduced to remove variants (i.e., miR-181b → miR-181) for the sake of simplicity, since the letters indicate differences in similar miRNA sequences within a species.

RESULTS AND DISCUSSION

This is the first study of its kind to identify sncRNA sequences potentially critical to supporting long-term vertebrate anoxia tolerance, through an intraspecific comparative approach. For a complete catalog of sequences identified in this study see Supplemental Table S3. Below, we discuss the potential importance of both constitutively expressed sncRNAs and anoxia-induced sncRNAs that may support anoxia tolerance in *A. limnaeus*. Importantly, many of the responses to anoxia are stage specific, even for stages with similar levels of anoxia tolerance. Though many unique sequences were identified, the discussion focuses on patterns of known stress-responsive miRNAs, as well as a subset of the potentially novel sncRNAs and miRNAs that may play critical roles in anoxia tolerance.

Total RNA levels are stabilized during anoxia in all stages of development. Total RNA per embryo differs by stage, increasing as the embryo develops (Fig. 2*A*), but is not altered by exposure to anoxia (Fig. 2*B*). This result is consistent with observations in other anoxia-tolerant organisms such as *Artemia* (45, 46) but contrasts with results for RNA yield in the telencephalon and ventricle of western painted turtles, *Chrysemys picta bellii,* exposed to 24 h of anoxia at 19°C (52). Each embryonic stage studied in *A. limnaeus* generated roughly equal numbers of raw reads, indicating comparable coverage (Table 1, Supplemental Table S1). Additionally, the number of unique small RNA reads in the catalog does not differ significantly by stage or by treatment (data not shown), justifying further comparison of the sncRNA expression profiles between stages and treatments.

Table 1. *Total number of reads for each developmental stage that contribute to the catalog of sncRNAs for Austrofundulus limnaeus*

Stage	Raw Reads	Trimmed Reads	Length Filtered Reads	Annotated Reads	Catalog of Unique sncRNAs
D2	280,731,104	226,858,108	167,944,096	109,525,916	413,534
4 dpd	280,160,813	208,773,716	159,493,842	124,875,018	565,883
12 dpd	224,773,200	140,786,180	122,722,291	98,305,042	411,782
20 dpd	300,226,485	214,093,118	186,984,753	130,533,583	346,533

D2, diapause 2; dpd, days postdiapause 2.

Stress-responsive miRNAs are highly expressed in all developmental stages. At each embryonic stage, many of the top 100 most abundant sncRNAs annotate to known stress-responsive miRNAs. Many of the sequences enriched in 4 dpd embryos, relative to D2 embryos, are also abundant in 12 dpd embryos (Fig. 3*A*, *clusters 1* and *4*); 68 of the 100 most highly expressed sequences in 4 dpd embryos are also highly expressed in 12 dpd and/or 20 dpd embryos (Fig. 3*B*). Of these sequences, ~63% (43 of 68) annotate to known stress-responsive miRNAs (Supplemental Table S4). Dormant (D2) embryos have a unique sncRNA transcriptome (see below) and only share 18 of their most abundant sncRNA sequences with post-D2 stages (Fig. 3*B*, Table 2). miRNAs with known stress-responsive function account for nearly all of these shared sequences. This result suggests that baseline expression of stress-responsive sncRNAs may predispose *A. limnaeus* embryos to tolerate stress, given that D2 embryos are the most stress-tolerant developmental stage of *A. limnaeus* (73). Despite enrichment of known sequences documented to respond to stress, these miRNAs may also have different roles. A single miRNA can target multiple genes and consequently have many biological functions. Several variants of miR-10b and miR-92a are abundant in each embryonic stage (Table 2), and these sequences are often dysregulated in cancer but also have normal functions in development (63, 69). Dissecting the function of such miRNAs in a general stress-response in *A. limnaeus* requires detailed experiments, including the identification of mRNA targets.

Stage- and tolerance-specific patterns of sncRNA abundance. Each embryonic stage, unique in its anoxia tolerance and physiology (Fig. 1), displays a distinct sncRNA profile under normoxic conditions (Fig. 3, Supplemental Tables S4 and S5, Supplemental Fig. S2) and in response to anoxia (Fig. 4, Supplemental Table S6, Supplemental Fig. S2). Evaluation of these expression patterns and the known differences in physiology, anoxia tolerance, and metabolic profiles of the stages allows for the identification of sequences that may be adaptive for surviving anoxia and should be targeted for detailed study in the future.

Table 2. *Sequences shared with D2 embryos that are present in the top 100 most abundant sequences in each post-D2 stage*

Sequence	Annotation	Function	D2	4 dpd	12 dpd	20 dpd
ACCCTGTAGAACCGAATTTGC	mir-10b	S	X	X	X	X
ACCCTGTAGAACCGAATTTGT	mir-10b	S	X	X	X	X
TACCCTGTAGAACCGAATTTG	mir-10b	S	X	X	X	X
TACCCTGTAGAACCGAATTTGC	mir-10b	S	X	X	X	X
TACCCTGTAGAACCGAATTTGT	mir-10b	S	X	X	X	X
TACCCTGTAGAACCGAATGTG	mir-10d	S	X	X	X	X
TACCCTGTAGAACCGAATGTGT	mir-10d	S	X	X	X	X
AACATTCAACGCTGTCGGTGA	mir-181a	S,H,IP	X	X	X	X
TATTGCACTTGTCCCGGCCTGTA	mir-92a-1	S,H,IP	X	X	X	X
TATTGCACTTGTCCCGGCCTGT	mir-92a-1/a-2	S,H,IP	X	X	X	X
TATTGCACTTGTCCCGGCCTG	mir-92a-1/a-2	S,H,IP	X	X	X	X
ACCCTGTAGAACCGAATTTG	mir-10b	S	X	X		
ACCCTGTAGAACCGAATGTGT	mir-10d	S	X	X		
AAGCTGCCAGCTGAAGAACT	mir-22a	S,H	X	X		
CAAGTGCTACACGTTGGGGTG	unknown	unknown	X	X		
CAAGTGCTACACGTTGGGGTGA	unknown	unknown	X	X		
TTTGGCAATGGTAGAACTCAC	mir-182	IP	X			X
AACATTCAACGCTGTCGGTG	mir-181a-1/a-2	S, H, IP	X			X

S, stress responsive; H, hypoxia responsive; IP, ischemic preconditioning responsive.

D2 embryos are uniquely poised for anoxic survival. Dormant D2 embryos express unique sncRNA patterns compared with the actively developing post-D2 stages. Expression of many D2-specific sequences (Fig. 3*A*, *clusters 2* and *3*) before exposure to anoxia fits with the unique physiological state (profound metabolic and developmental arrest) of D2 embryos (77). These D2-specific sequences may support regulation of metabolism, protein synthesis, and cell cycle arrest associated with entrance into D2 (44, 73, 74). They may also play a role in support of anoxia tolerance as D2 embryos appear prepared for anaerobic metabolism even under aerobic conditions, and thus few adjustments are needed during their entry into anoxia (25, 30, 77).

D2 embryos may be prepared at baseline with a high abundance of sequences required to regulate the stressful

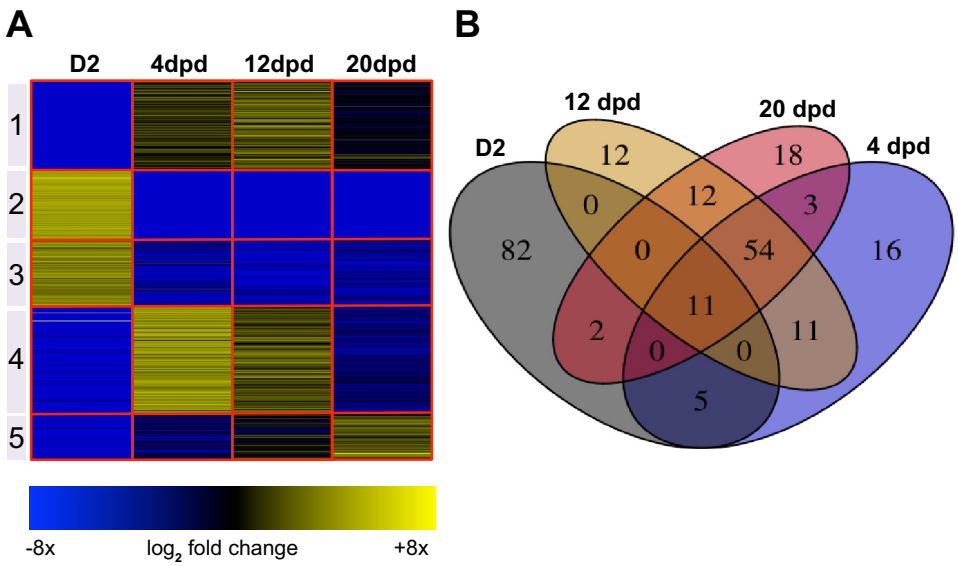

Fig. 3. sncRNA abundance as a function of developmental stage in normoxia. *A*: heat map of mean abundance values for sncRNAs that are highly differentially expressed (adjusted *P* value < 0.01, $\log_2$ fold change > 2, and normalized mean expression across all samples > 25) between developmental stages in normoxia ($t = 0$). $\log_2$ fold change values are calculated relative to mean expression in D2 embryos. Yellow indicates increased expression relative to the D2 mean, while blue indicates decreased expression. Gray indicates a missing value due to the absence of that sncRNA in that developmental stage. Expression patterns were parsed into 5 basic clusters by K-means clustering. See Supplemental Table S5 and Supplemental Fig. S1 for detail on these sequences. *B*: Venn diagram of top 100 most abundant sequences expressed in normoxic embryos ($t = 0$). See Table 2 for details on these abundant sequences that are shared in D2 and post-D2 embryos and Supplemental Table S4 for details on all the top 100 most abundant sequences.

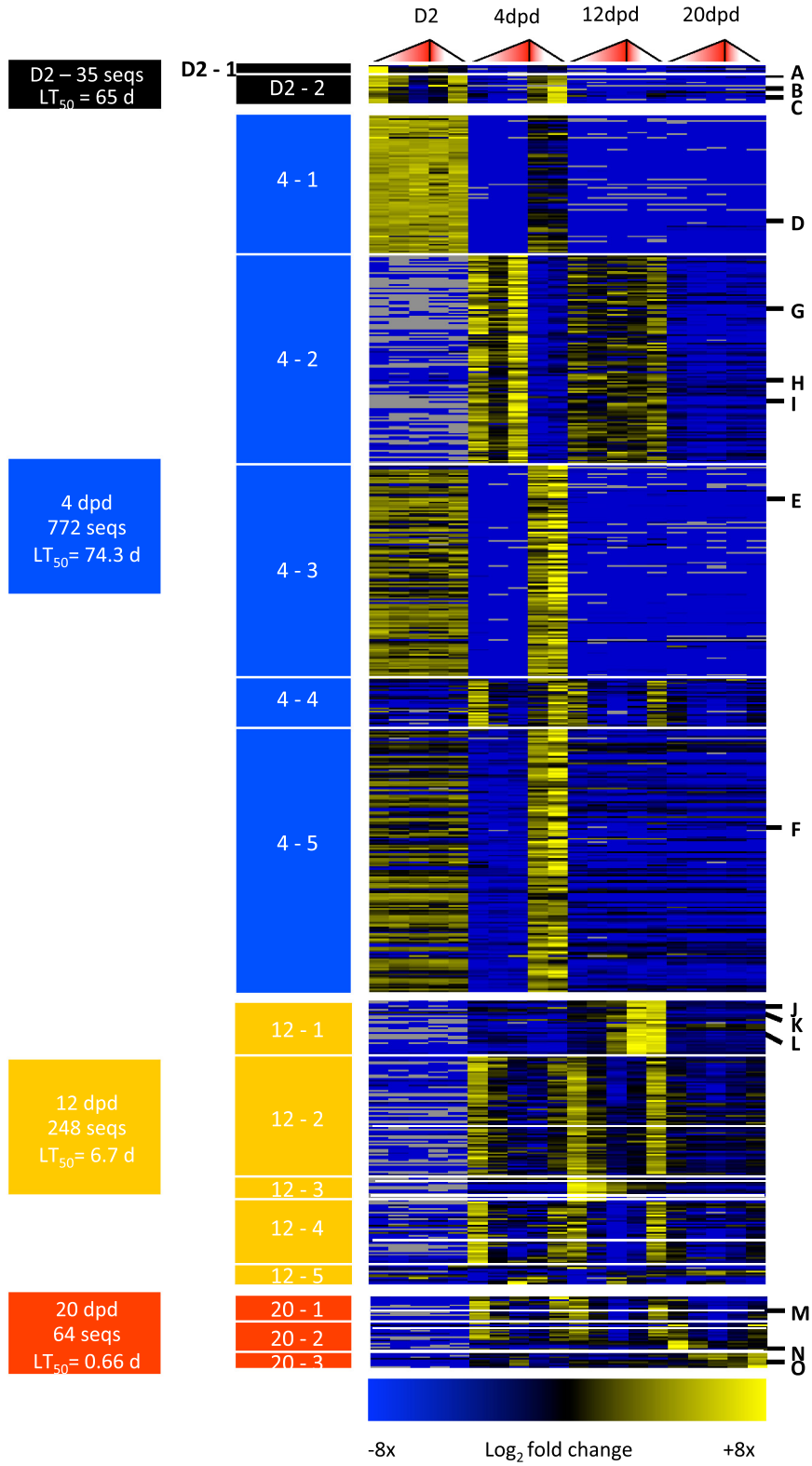

Fig. 4. Heat map of sncRNA differential expression in response to anoxia and recovery from anoxia in each developmental stage. Highly differentially expressed sncRNAs (adjusted P value < 0.01, $\log_2$ fold change > 2, and normalized mean expression across all samples > 25) were clustered within each stage (cluster numbers are listed on the *left* of the heat map). Within each cluster, expression patterns are displayed for all stages, even though expression may not be statistically different in the other stages. Because differential expression was determined within each stage, some sncRNA sequences may be represented in multiple stages. Within each stage, expression on the heat map corresponds with exposure to anoxia and recovery from anoxia (indicated by the gradient filled triangles above the heat map; from *left* to *right*: $t = 0$ (normoxia), early anoxia, late anoxia, early recovery, late recovery. For details on sampling see Fig. 1. Log_2 fold change values were calculated relative to the mean expression of all sncRNAs over all 80 samples (all replicates of all stages and treatments). Yellow indicates increased expression relative to the mean while blue indicates decreased expression. Gray indicates a missing value due to absence of that sequence in that experimental treatment. Letters to the *right* of the heat map indicate sequences that are represented in line graphs in Fig. 6. See Supplemental Table S6 and Supplemental Fig. S2 for detail on these and other differentially expressed sequences.

physiological transition associated with reoxygenation. The normoxic D2 sncRNA transcriptome is enriched for sequences that increase in abundance during recovery from anoxia in metabolically active anoxia-tolerant 4 dpd embryos (Fig. 4, *clusters 4-1, 4-3, 4-5*). In fact, 96% of the sncRNAs in Fig. 4, *cluster 4-1,* are abundant at $t = 0$ in D2 embryos (Fig. 3, *clusters 1* and *2*). Many (40%) of the recovery-responsive 4 dpd sequences annotate to known stress-responsive sncRNAs. The high proportion of these sequences constitutively expressed in D2 embryos may prepare them for a successful transition out of anoxia without the need for de novo synthesis of sncRNAs critical for this process.

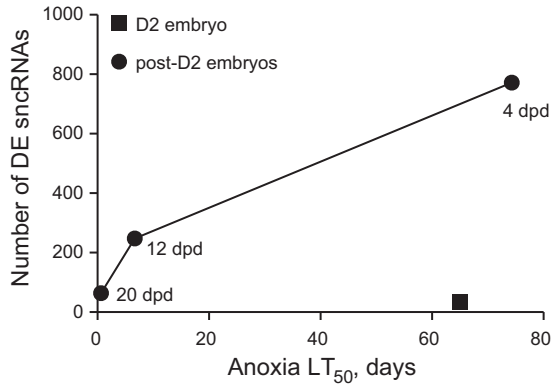

Fig. 5. The relationship between anoxia tolerance and the number of highly differentially expressed (DE) sequences for each stage. Note that D2 embryos have a low number of differentially expressed sncRNAs, even though they have a high tolerance of anoxia.

D2 embryos lack a robust sncRNA transcriptomic response to anoxia, with only 35 sncRNA sequences changing in abundance. In contrast, for post-D2 stages those with higher tolerances of anoxia tend to have a greater number of differentially expressed sncRNAs (Fig. 5). This lack of a large-scale sncRNA transcriptomic response in D2 embryos is consistent with the intrinsically low metabolic rate and limited capacity for gene expression associated with diapause (78) (Fig. 1). Of the few anoxia-responsive sncRNAs identified in D2 embryos, over half increase in abundance in 4 dpd embryos (extremely anoxia-tolerant, metabolically active embryos) during recovery from anoxia (Fig. 4, *groups B* and *C*). This pattern again suggests preparation in D2 embryos for reoxygenation as described above for constitutively expressed sncRNAs.

In D2 embryos, most anoxia-responsive sncRNAs decrease in abundance during anoxia and then return to baseline levels during recovery (Fig. 4, *cluster D2-2*; Fig. 6, *B* and *C*). We hypothesize that the decreased abundance of these miRNAs suggests that they are consumed as they work to alter translational efficiency of proteins necessary for surviving entrance into anoxia. Alternatively, these miRNAs could be blocking translation of mRNA transcripts during dormancy, and thus degradation or decreased synthesis of the miRNA could allow for immediate translation of the mRNAs in response to anoxia. Identifying the targets of these miRNAs may help us identify genes required to tolerate anoxia.

Only a handful of sequences in D2 embryos increase in abundance during anoxia and return to nearly undetectable levels during recovery (Fig. 4, *cluster D2-1*; Fig. 6*A*). These sequences may decrease translation of proteins required under oxygenated conditions that are incompatible with the biochemical requirements for surviving anoxia. Beyond the function of these miRNAs, understanding how the embryo generates them when cellular energy stores (ATP levels) are especially low (81) may reveal unique aspects of sncRNA synthesis in this species.

4 dpd embryos display large and diverse changes in sncRNA abundance in response to anoxia and recovery, relative to other post-D2 stages. In extremely anoxia-tolerant 4 dpd embryos, exposure to anoxia significantly alters the abundance of 772 sncRNAs (Figs. 4 and 5), suggesting that survival of anoxia may therefore require these changes in sncRNA abundance levels. Exposure to anoxia induces two major patterns in sncRNA expression in 4 dpd embryos. First, many sncRNAs increase in abundance dramatically during recovery (Fig. 4, *clusters 4-1, 4-3, 4-5*). These are the same clusters referenced above that are constitutively expressed in D2 embryos, but of low abundance or not expressed in normoxic post-D2 stages (Fig. 6, *A–C*). Thus, it seems that highly anoxia-tolerant and metabolically active (4 dpd) embryos must produce sncRNA sequences de novo during recovery, which highly anoxia-tolerant and dormant embryos (D2) express in abundance regardless of exposure to anoxia. Based on this expression we hypothesize that these sequences support homeostasis during the critical transition from anoxia to normoxia in 4 dpd embryos.

Dramatic changes in sncRNA abundance during recovery is not surprising given that successful transition between anoxia and normoxia is essential for survival, but particularly challenging from a cell biological perspective (11). Reperfusion injury occurs when restoration of circulation delivers a surge of oxygen to tissues and increases the chances of reactive oxygen species (ROS) production (19). ROS damages DNA, cell membranes, and proteins (90) and therefore threatens survival. Recent discovery of miRNAs that regulate ROS production (48) and sense redox changes (redoximiRs) (24) supports the idea that these recovery-responsive miRNAs could be involved in preventing or ameliorating reperfusion injury. In 4 dpd embryos exposed to anoxia, 17 miRNAs previously reported to play a role in regulating and responding to ROS (Table 3) increase significantly in abundance during recovery, relative to normoxic ($t = 0$) levels (Fig. 4, *clusters 4-1, 4-3, 4-5*). Therefore, we hypothesize that the recovery-responsive miRNAs overwhelming the sncRNA response to anoxia in 4 dpd embryos are involved in regulating ROS and mitigating risks of reperfusion injury required for survival of long-term anoxia. Additionally, our data may contain novel ROS-responsive and ROS-regulating sequences yet to be discovered in other systems.

Long-term anoxia tolerance. From a metabolic perspective, it is difficult to distinguish a normoxic D2 embryo from an anoxic one, probably because D2 embryos are already dormant when exposed to anoxia (81). In stark contrast, embryos at 4 dpd are metabolically active when exposed to anoxia and quickly depress their metabolism and enter a state of quiescence (81). Despite their divergent physiological states, D2 and

Fig. 6. Normalized expression of the most significantly differentially expressed (based on *P* value) sequences in each developmental stage (see Fig. 4). Normalized expression (means ± SE) for all 4 stages is presented for each sequence. Solid lines indicate time during anoxia, while dotted lines denote aerobic recovery. *A, B, C*: sequences differentially expressed in D2 embryos. *D, E, F*: sequences with increased expression during recovery in 4 dpd embryos. *G, H, I*: mitosRNAs derived from tRNA fragments that increase in abundance during anoxia in 4 dpd embryos. *J, K, L*: sequences that are putatively anoxic preconditioning responsive in 12 dpd embryos. *M, N, O*: expression patterns of sequences for 20 dpd embryos. EA, early anoxia; LA, late anoxia; ER, early recovery; LR, late recovery. See Fig. 1 for sampling times that correspond to these samples.

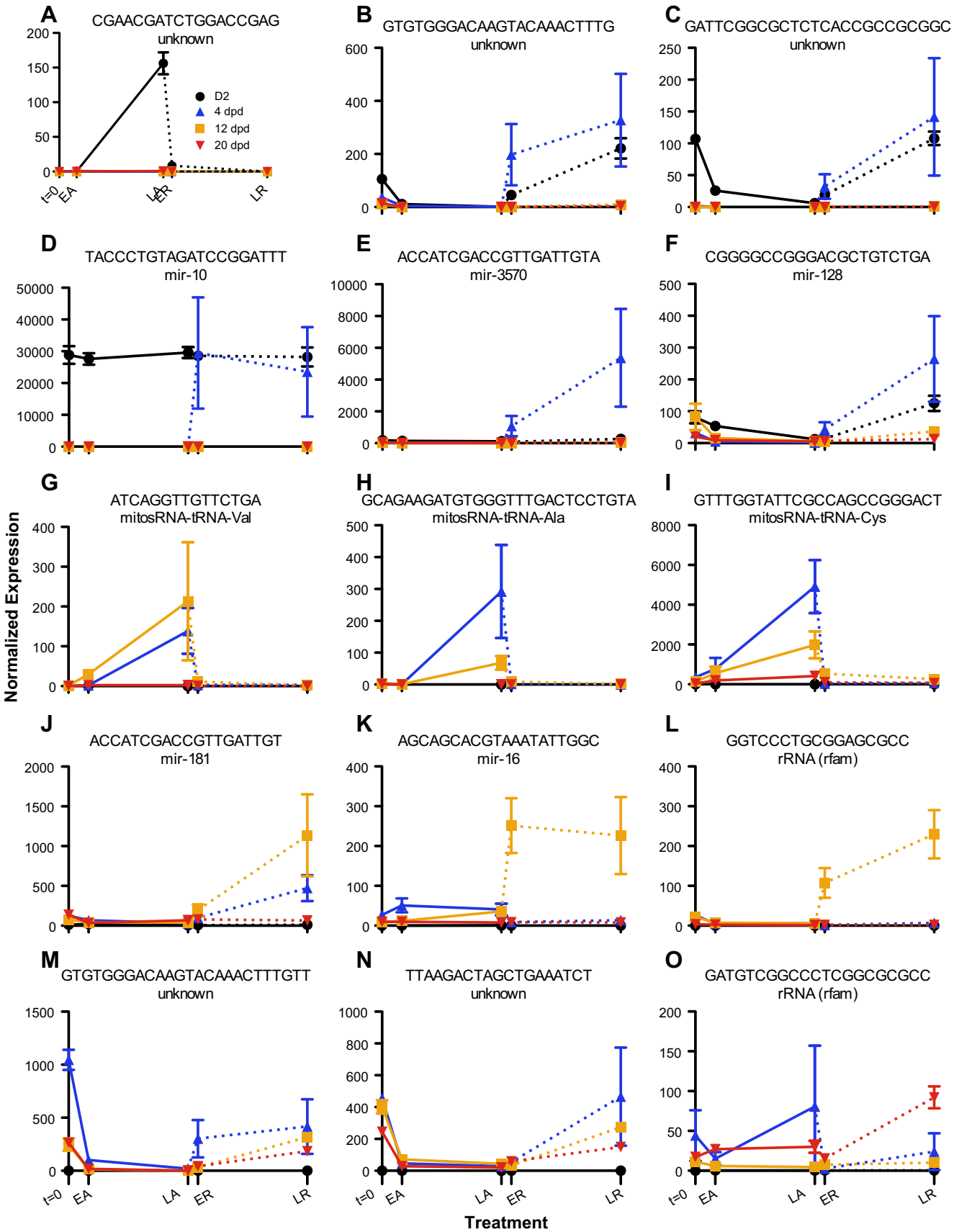

Table 3. *Recovery-responsive sncRNAs in 4 dpd embryos annotate to miRNAs known to regulate reactive oxygen species physiology*

Sequence	Annotation	Function Documented in Literature	Cluster
AGGATATCATCTTATACTGTAA	mir-144	regulate generation of ROS*	4–5
AGGCGGAGACTTGAGCAATT	mir-25	regulate generation of ROS*	4–5
AGGCGGAGACTTGAGCAATTG	mir-25	regulate generation of ROS*	4–5
ATCACATTGCCAGGGATTA	mir-23b	regulate generation of ROS*	4–1
ATCACATTGCCAGGGATTAC	mir-23b	regulate generation of ROS*	4–5
ATTGCACTTGTCTCGGTCT	mir-25	regulate generation of ROS*	4–5
CATTGCACTTGTCTCGG	mir-25	regulate generation of ROS*	4–5
CATTGCACTTGTCTCGGT	mir-25	regulate generation of ROS*	4–1
CATTGCACTTGTCTCGGTC	mir-25	regulate generation of ROS*	4–5
CATTGCACTTGTCTCGGTCT	mir-25	regulate generation of ROS*	4–5
GGATATCATCTTATACTGTAA	mir-144	regulate generation of ROS*	4–5
TAACACTGTCTGGTAACGA	mir-200a	affect ROS levels in cancer cells†	4–5
TAACACTGTCTGGTAACGAT	mir-200a	affect ROS levels in cancer cells†	4–5
TAGGTAGTTTCATGTTGTTG	mir-196	regulate Nrf2 and related regulatory proteins*	4–3
TGAGAACTGAATTCCATAGAT	mir-146a	regulate generation of ROS*	4–3
TTAATGCTAATCGTGATAGGG	mir-155	regulate Nrf2 and related regulatory proteins*	4–5
TTGCATAGTCACAAAAGTGA	mir-153	regulate Nrf2 and related regulatory proteins*	4–3

Cluster, differential expression cluster as presented in Fig. 4. *Cheng et al. 2013 (24); †He and Jiang 2016 (48).

4 dpd embryos survive anoxia for about the same about of time (Fig. 1). Consistent with their differences in physiology, their sncRNA profiles and responses to anoxia are distinct. However, the constitutively expressed sncRNA transcriptome of D2 embryos is very similar to the recovery-specific transcriptome of 4 dpd embryos (see above). Furthermore, these sequences may specifically support the survival of long-term anoxia, as they are absent or not differentially expressed in older, less anoxia-tolerant embryos (12 and 20 dpd).

Mitochondrial sncRNA transcriptome responds to anoxia. During anoxia, extremely anoxia-tolerant 4 dpd embryos deplete and regain transcripts of a unique class of sncRNAs (Fig. 4, *cluster 4-2*). These sequences initially decrease in abundance after 4 h of anoxia, compared with baseline levels, but drastically increase in abundance by 24 h of anoxia (Fig. 6, *G–I*). The same sequences display similar expression patterns in 12 dpd embryos, but for the most part only in 4 dpd embryos does the change in abundance rise to the level of statistical significance. In contrast to the above discussion, these sequences are not abundant during recovery. This pattern suggests possible degradation or consumption of the sequences during transitions into and out of anoxia. The few published studies on sncRNA expression during anoxia that include time-course analyses generally show a graded increase or decrease in expression as the anoxic bout continues (15, 95). Synthesis of these sequences during anoxia is surprising since the lack of oxygen adversely affects the canonical miRNA biogenesis pathway (70). However, most of these sequences are not similar to any known miRNAs but, rather, map to the mitochondrial genome of *A. limnaeus.* As noncanonical miRNAs, their generation may be possible under anoxia. The 4 dpd anoxia-responsive cluster (Fig. 4, *cluster 4-2*) is particularly enriched for sequences derived from mitochondrial transfer-RNAs (tRNAs) (Fig. 7). While a few mitochondria-derived sequences increase in abundance during recovery from anoxia (Fig. 4, *clusters 4-3* and *4-5*), mitochondria-derived sequences comprise the majority (75%) of sncRNAs that increase in abundance during anoxia in 4 dpd embryos. This unique signature of anoxia-responsive mitochondria-derived sncRNAs characterizes 4 dpd embryos, the most anoxia-tolerant and metabolically active

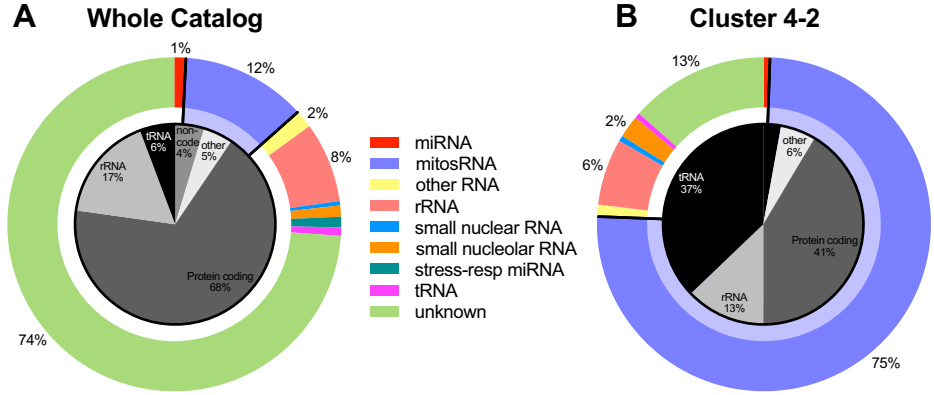

Fig. 7. Distribution of the types of sncRNAs identified in embryos of *A. limnaeus*. *A*: the outer ring (colors) represents the percent of each annotation category for all sncRNAs identified in the complete catalog (any stage and treatment) of *A. limnaeus*. The inner pie chart (grayscale) represents the annotation location within the mitochondrial genome for mitosRNAs identified in the complete *A. limnaeus* sncRNA catalog. *B*: the outer ring (colors) represents sncRNAs that were highly abundant after 24 h of anoxia in 4 dpd embryos (Fig. 4, *cluster 4-2*). The inner pie chart (grayscale) represents the annotation location within the mitochondrial genome for mitosRNAs present in this cluster. Note the enrichment of tRNA-derived sequences in the highly differentially expressed cluster compared with their representation in the whole catalog.

stage. We hypothesize that these mitochondria-derived sncRNAs may be central to the development of extreme anoxia tolerance in active *A. limnaeus* embryos.

Mitochondria-derived small RNAs (mitosRNAs) (84) and mitomiRs (miRNAs encoded in the nuclear genome and localized to mitochondria) (8, 29, 94) have recently been described, and some mitomiRs have even been associated with hypoxia and metabolic function (94). However, this is the first report, to our knowledge, of stress-responsive mitosRNAs. Furthermore, the enrichment of tRNA-derived sequences could have implications for regulation of protein synthesis, which is commonly suppressed during anoxia-induced quiescence (57), by taking mitochondrial tRNAs out of commission. It is therefore plausible that tRNAs are selectively processed to support metabolic depression and were coopted for further function to support anoxia tolerance in *A. limnaeus* via regulation of gene expression. Conversely, distinct mechanisms within the mitochondrion may synthesize these sncRNAs under anoxia. The presence of some mitosRNAs with several bases on the 5′-end that are not modeled to exist in the mature tRNA sequences supports the biosynthesis hypothesis of these sequences during anoxia (as opposed to degradation of existing tRNAs), indicating that their production may not be tRNA degradation, but alternative processing of RNA transcripts. However, it is possible that these models are incorrect, and detailed sequencing of mature tRNAs will be needed to clarify the pathway by which these sncRNAs are created.

Given the central importance of mitochondrial physiology to oxygen sensing and regulation of programmed cell death pathways in response to anoxia and ischemia (92), we hypothesize that this group of mitochondria-derived anoxia-responsive sncRNAs are essential for maintaining mitochondrial homeostasis and function in metabolically active embryos during transitions into and out of anoxia (D2 embryos do not express these sequences in high abundance). This novel finding may open new avenues for understanding how mitochondrial function supports anoxia tolerance and how to preserve its function in the face of anoxia.

The induction of mitosRNAs in response to anoxia, observed in 4 dpd embryos, has not been previously described and therefore may be species specific. However, most sncRNA studies focus on the role of conserved miRNAs, and therefore mitochondria-derived sncRNA sequences may have simply been overlooked in other studies. Data mining of deep sequencing small RNA projects is an important next step in exploring a more generalized role for mitosRNAs in the support of stress tolerance.

12 dpd embryos express putative preconditioning-responsive sncRNAs. Preconditioning 12 dpd embryos with 24 h of anoxia followed by 24 h of aerobic recovery extends anoxia tolerance by over 30% (Fig. 1) (82). Thus, 12 dpd-specific sequences that increase in abundance during recovery from anoxia are induced by the preconditioning regime and may play a role in extending anoxia tolerance. While most of the sncRNAs differentially expressed over exposure to anoxia and recovery in preconditioning-responsive 12 dpd embryos are more abundant during recovery, only one cluster comprises sequences that dramatically increase in expression at 2 and 24 h of recovery (Fig. 4, *cluster 12-1*). Additionally, anoxia does not induce differential expression of these sequences in any other embryonic stage, suggesting that they may be specific to the preconditioning phenotype (Fig. 6, *J–L*). Based on this expression pattern and the preconditioning-responsive physiology of 12 dpd embryos, we hypothesize that these sequences may support extended survival of anoxia following preconditioning. Furthermore, the majority (85%) of these sequences annotate to known stress-responsive miRNAs, a broad category including hypoxia, preconditioning, and stress/hypometabolism-responsive sequences (Table 4). Of these sequences, over half (54%) annotate to known preconditioning-responsive miRNAs, but only one of the sequences (mir-153) is solely documented as preconditioning-responsive in the literature. The majority of these putative preconditioning-responsive sequences respond to either stress/hypometabolism or a variety of stresses including hypoxia, and preconditioning. This overlap led us to hypothesize that preconditioning may activate a robust conserved general stress response in *A. limnaeus* embryos that supports survival of subsequent exposures to anoxia. Hypoxia-, ischemia-, and preconditioning responsive sncRNAs from mammalian species are not represented in large numbers in these sequences, indicating that the preconditioning response in *A. limnaeus*, a highly anoxia-tolerant species, may be distinct from that of anoxia-sensitive mammals.

Anoxia-sensitive 20 dpd embryos do not mount a robust sncRNA response to anoxia. Metabolically active embryos near hatching and sensitive to anoxia (20 dpd, Fig. 1) fail to mount a robust sncRNA response to anoxia. Exposure to anoxia only induces changes in expression of 64 sequences in these embryos, compared with 224 and 772 sequences in 12 dpd and 4 dpd embryos, respectively (Figs. 4 and 5). Of the few sequences that change in abundance about half are shared with those differentially expressed in 12 dpd embryos (Fig. 4, *clusters 20-1* and *20-2*). In general, these sequences are less abundant in 20 dpd embryos than in other post-D2 embryos, indicating a muted response (Fig. 6, *M–O*). A diminished sncRNA response to anoxia in 20 dpd embryos, relative to other post-D2 embryos, may underlie the reduced anoxia tolerance and limited ability to suppress metabolism observed in these prehatching embryos. In contrast to the anoxia-tolerant 4 dpd embryos, the heart continues to beat during anoxia in embryos 12 dpd and older (33; J. E. Podrabsky, personal observation). We therefore hypothesize that the anoxia-sensitivity of 20 dpd embryos may be linked to their inability to mount a robust sncRNA response that regulates biochemical and physiological changes required for long-term anoxic survival.

In 20 dpd embryos, anoxia induces two distinct sequence expression patterns (Fig. 4 *clusters 20-2* and *20-3*). First, sequences abundant under normoxia decline in abundance and do not regain normoxic levels within 24 h of recovery (Fig. 4, *cluster 20-2*). Of the seven sequences with this distinct pattern, four have not previously been described and three annotate to variants of mir-1b. Mir-1 variants are associated with ischemic preconditioning (91) and hypometabolism (22, 42) (Supplemental Table S2). Recovering from anoxia produces an increase in transcript abundance of a second group of sncRNAs in 20 dpd embryos (Fig. 4, *cluster 20-3*). Many of these sequences are also recovery responsive in extremely anoxia-tolerant 4 dpd embryos (Fig. 6*O*). The significance of these expression patterns remains unclear, but the generally reduced response in anoxia-sensitive 20 dpd embryos compared with

Table 4. *Preconditioning-responsive sncRNAs annotate to known stress-responsive miRNAs in 12 dpd embryos*

miRNA Class	*A. limnaeus* Sequence	Annotation	Function in Literature	Stress Conditions
mir-16	AGCAGCACGTAAATATTGGAG	mir-16b	stress (12, 95, 105, 109), hypoxia (54)	freezing (frog), hibernation (marsupial), anoxia (turtle), dehydration
	AGCAGCACGTAAATATTGGCG	mir-16-1/2		
	AGCAGCACGTAAATATTGGC (Fig. 6*K*)	mir-16-1/2		
mir-18	AAGGTGCATCTAGTGTAGTTG	mir-18	stress (16, 42, 109)	hibernation (bat, marsupial)
mir-22	AGCTGCCAGCTGAAGAACTG	mir-22a	stress (23, 42, 109), hypoxia (4)	hibernation (marsupial), aestivation (sea cucumber), hibernation (bat)
	AGCTGCCAGCTGAAGAACTGT	mir-22a		
	AGCTGCCAGCTGAAGAACT	mir-22a		
mir-222	GCTCAGTAGTCAGTGTAGATCC	mir-222a	stress (22, 106, 109)	hibernation (ground squirrel, bat), aestivation (sea cucumber)
mir-26	TCAAGTAATCCAGGATAGGCTT	mir-26a-1/3	stress (7, 95), hypoxia (54)	hibernation (marsupial), freezing (frog)
	TCAAGTAATCCAGGATAGGCT	mir-26a-1/2		
	TCAAGTAATCCAGGATAGGTT	mir-26b		
mir-27	TCACAGTGGCTAAGTTCTGCA	mir-27b	stress (7, 95, 109), hypoxia (54), preconditioning (28)	hibernation (marsupial, bat), freezing (frog)
	TCACAGTGGCTAAGTTCTGC	mir-27b		
	TCACAGTGGCTAAGTTCTG	mir-27b		
	TCACAGTGGTTAAGTTCTGCC	mir-27c		
	TCACAGTGGTTAAGTTCTGC	mir-27c-1/2		
	TCACAGTGGTTAAGTTCTG	mir-27c-1/2		
	TCACAGTGGCTAAGTTCAGT	mir-27e		
mir-30	GTAAACATCCTACACTCAGCT	mir-30b	stress (7, 64), hypoxia (54), preconditioning (28)	freezing (frog), dehydration (frog)
	GTAAACATCCTACACTCTCAGCT	mir-30c-2/1		
	GTAAACATCCTACACTCTCAGC	mir-30c-2/1		
	GTAAACATCCCCGACTGGAAGCT	mir-30d		
	GTAAACATCCCCGACTGGAAGC	mir-30d		
	GTAAACACCCTACACTCTCGGC	mir-30f		
mir-140	CCACAGGGTAGAACCACGGAC	mir-140	stress (7), hypoxia (18), preconditioning (28)	freezing (frog)
mir-143	GAGATGAAGCACTGTAGCTC	mir-143	stress (106)	hibernation (ground squirrel)
	GAGATGAAGCACTGTAGCT	mir-143		
mir-153	TTGCATAGTCACAAAAGTG	mir-153	preconditioning (28)	
mir-181	CCATCGACCGTTGATTGT	mir-181a-1	stress (7, 16, 64, 95), hypoxia (54), preconditioning (28)	hibernation (marsupial), dehydration (frog), freezing (frog)
	CCATCGACCGTTGACTGTACC	mir-181a-2		
	ACCATCGACCGTTGATTGT (Fig. 6*J*)	mir-181a-1		
	ACCATCGACCGTTGACTGT	mir-181a-1		
	ACATTCAACGCTGTCGGTGAGT	mir-181a-2/1		
	ACATTCAACGCTGTCGGTGAG	mir-181a-2/1		
	ACATTCAACGCTGTCGGTGA	mir-181a-2/1		
mir-183	TGGCACTGGTAGAATTCACTGT	mir-183	stress (16), preconditioning (60)	hibernation (bat)
mir-199	CCAGTGTTCAGACTACCTGTTC	mir-199b-2	stress (7), hypoxia (18), preconditioning (108)	freezing (frog)
mir-204	TCCCTTTGTCATCCTATGCCT	mir-204	hypoxia (27), preconditioning (28)	
mir-222	GCTCAGTAGTCAGTGTAGATCC	mir-222a	stress (22, 106, 109)	hibernation (ground squirrel, bat), aestivation (sea cucumber)
mir-429	AATACTGTCTGGTAATGCCGT	mir-429	hypoxia (54), preconditioning (60)	
mir-455	ATGTGCCCTTGGACTACATCG	mir-455-1	stress (7, 109)	freezing (frog), hibernation (bat)

For "Function in Literature" see Supplemental Table S1 for more information.

anoxia-tolerant post-D2 stages suggests a reduced capacity to respond to anoxia via alterations in the sncRNA transcriptome.

Limitations and Challenges

This study provides a global overview of sncRNA identity and abundance in a species with anoxia-sensitive and anoxia-tolerant phenotypes, allowing us to identify compelling groups of sequences to further investigate. However, as a whole-embryo study, this work lacks organ- and cell-type specific expression information. sncRNAs differentially expressed only in a specific tissue or cell type may also go undetected in this study, as the abundance of sequences from other tissues dwarfs their expression. Comparison with known stress-responsive miRNAs relies on imperfect methods, assuming functional similarity between sequences annotating to the same miRNAs active in distinct species. Additionally, since *A. limnaeus* is emerging as a genomic and transcriptomic model and we have chosen to consider all sncRNAs, identifying targets and functions remains a significant challenge. However, identifying novel ways to prevent tissue damage as a consequence of oxygen deprivation or to engineer vertebrate cells to survive without oxygen remains a rewarding challenge.

Conclusions and Future Directions

In conclusion, comparing sncRNA profiles of distinct phenotypes within *A. limnaeus* has yielded a number of expected and surprising outcomes, with the identification of known hypoxia-responsive miRNAs as well as novel sncRNAs. The intraspecific comparative approach proved powerful for identifying unique sncRNA sequences that, based on expression

pattern, we predict mediate various aspects of anoxic biology in this species. Robust differences in sncRNA expression patterns exhibited by each unique developmental stage allowed for the identification of sncRNA sequences that, with further focused study, may transform our understanding of vertebrate anoxia tolerance. In particular, this study generated great interest and focus on mitosRNAs, which almost certainly would not have been identified had our study been limited to one embryonic stage or to evaluation of known miRNAs. To examine the biology of mitosRNAs, we will probe their location and test their function in whole embryos and cell culture derived from anoxia-tolerant *A. limnaeus* embryos. These cell culture studies will provide greater resolution, spatially and functionally, and allow us to examine proteomic changes associated with over- or underexpression of sncRNAs of interest. In addition, existing sncRNA data for other organisms must be searched for similar stress-responsive mitosRNAs, in both anoxia-tolerant and anoxia-sensitive species. These comparative genomic and transcriptomic studies could help identify common and adaptive responses to anoxia in vertebrates. In addition, these studies may help to distinguish, in anoxia-sensitive species, adaptive responses from those due to dysregulation in the face of cellular stress due to oxygen deprivation.

ACKNOWLEDGMENTS

We thank Will Patterson for help with bioinformatics programming and Amie Romney for assistance with generating the sncRNA data pipeline.

GRANTS

This work was funded by National Science Foundation (NSF) Grant IOS-1354549 to J. E. Podrabsky and NSF Graduate Research Fellowship DGE-1057604 to C. L. Riggs.

DISCLOSURES

The authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

C.L.R. and J.E.P. conceived and designed research; C.L.R. performed experiments; C.L.R. analyzed data; C.L.R. and J.E.P. interpreted results of experiments; C.L.R. prepared figures; C.L.R. drafted manuscript; C.L.R. and J.E.P. edited and revised manuscript; C.L.R. and J.E.P. approved final version of manuscript.

REFERENCES

1. **Ambros V.** The functions of animal microRNAs. *Nature* 431: 350–355, 2004. doi:10.1038/nature02871.
2. **Anders S, Huber W.** Differential expression analysis for sequence count data. *Genome Biol* 11: R106, 2010. doi:10.1186/gb-2010-11-10-r106.
3. **Anderson SN, Podrabsky JE.** The effects of hypoxia and temperature on metabolic aspects of embryonic development in the annual killifish *Austrofundulus limnaeus*. *J Comp Physiol B* 184: 355–370, 2014. doi:10.1007/s00360-014-0803-6.
4. **Azzouzi HE, Leptidis S, Doevendans PA, De Windt LJ.** HypoxamiRs: regulators of cardiac hypoxia and energy metabolism. *Trends Endocrinol Metab* 26: 502–508, 2015. doi:10.1016/j.tem.2015.06.008.
5. **Bandiera S, Matégot R, Girard M, Demongeot J, Henrion-Caude A.** MitomiRs delineating the intracellular localization of microRNAs at mitochondria. *Free Radic Biol Med* 64: 12–19, 2013. doi:10.1016/j.freeradbiomed.2013.06.013.
6. **Bandiera S, Rüberg S, Girard M, Cagnard N, Hanein S, Chrétien D, Munnich A, Lyonnet S, Henrion-Caude A.** Nuclear outsourcing of RNA interference components to human mitochondria. *PLoS One* 6: e20746, 2011. doi:10.1371/journal.pone.0020746.
7. **Bansal S, Luu BE, Storey KB.** MicroRNA regulation in heart and skeletal muscle over the freeze-thaw cycle in the freeze tolerant wood frog. *J Comp Physiol B* 186: 229–241, 2016. doi:10.1007/s00360-015-0951-3.
8. **Barrey E, Saint-Auret G, Bonnamy B, Damas D, Boyer O, Gidrol X.** Pre-microRNA and mature microRNA in human mitochondria. *PLoS One* 6: e20220, 2011. doi:10.1371/journal.pone.0020220.
9. **Bartel DP.** MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116: 281–297, 2004. doi:10.1016/S0092-8674(04)00045-5.
10. **Bartel DP.** MicroRNAs: target recognition and regulatory functions. *Cell* 136: 215–233, 2009. doi:10.1016/j.cell.2009.01.002.
11. **Bickler PE, Buck LT.** Hypoxia tolerance in reptiles, amphibians, and fishes: life with variable oxygen availability. *Annu Rev Physiol* 69: 145–170, 2007. doi:10.1146/annurev.physiol.69.031905.162529.
12. **Biggar KK, Dubuc A, Storey K.** MicroRNA regulation below zero: differential expression of miRNA-21 and miRNA-16 during freezing in wood frogs. *Cryobiology* 59: 317–321, 2009. doi:10.1016/j.cryobiol.2009.08.009.
13. **Biggar KK, Kornfeld SF, Maistrovski Y, Storey KB.** MicroRNA regulation in extreme environments: differential expression of microRNAs in the intertidal snail *Littorina littorea* during extended periods of freezing and anoxia. *Genomics Proteomics Bioinformatics* 10: 302–309, 2012. doi:10.1016/j.gpb.2012.09.002.
14. **Biggar KK, Storey KB.** The emerging roles of microRNAs in the molecular responses of metabolic rate depression. *J Mol Cell Biol* 3: 167–175, 2011. doi:10.1093/jmcb/mjq045.
15. **Biggar KK, Storey KB.** Evidence for cell cycle suppression and microRNA regulation of cyclin D1 during anoxia exposure in turtles. *Cell Cycle* 11: 1705–1713, 2012. doi:10.4161/cc.19790.
16. **Biggar KK, Storey KB.** Identification and expression of microRNA in the brain of hibernating bats, *Myotis lucifugus*. *Gene* 544: 67–74, 2014. doi:10.1016/j.gene.2014.04.048.
17. **Bolger AM, Lohse M, Usadel B.** Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30: 2114–2120, 2014. doi:10.1093/bioinformatics/btu170.
18. **Boon RA, Dimmeler S.** MicroRNAs in myocardial infarction. *Nat Rev Cardiol* 12: 135–142, 2015. doi:10.1038/nrcardio.2014.207.
19. **Braunersreuther V, Jaquet V.** Reactive oxygen species in myocardial reperfusion injury: from physiopathology to therapeutic approaches. *Curr Pharm Biotechnol* 13: 97–114, 2012. doi:10.2174/138920112798868782.
20. **Bueno MJ, Malumbres M.** MicroRNAs and the cell cycle. *Biochim Biophys Acta* 1812: 592–601, 2011. doi:10.1016/j.bbadis.2011.02.002.
21. **Carrington JC, Ambros V.** Role of microRNAs in plant and animal development. *Science* 301: 336–338, 2003. doi:10.1126/science.1085242.
22. **Chen M, Storey KB.** Large-scale identification and comparative analysis of miRNA expression profile in the respiratory tree of the sea cucumber *Apostichopus japonicus* during aestivation. *Mar Genomics* 13: 39–44, 2014. doi:10.1016/j.margen.2014.01.002.
23. **Chen M, Zhang X, Liu J, Storey KB.** High-throughput sequencing reveals differential expression of miRNAs in intestine from sea cucumber during aestivation. *PLoS One* 8: e76120, 2013. doi:10.1371/journal.pone.0076120.
24. **Cheng X, Ku CH, Siow RCM.** Regulation of the Nrf2 antioxidant pathway by microRNAs: New players in micromanaging redox homeostasis. *Free Radic Biol Med* 64: 4–11, 2013. doi:10.1016/j.freeradbiomed.2013.07.025.
25. **Chennault T, Podrabsky JE.** Aerobic and anaerobic capacities differ in embryos of the annual killifish *Austrofundulus limnaeus* that develop on alternate developmental trajectories. *J Exp Zool A Ecol Genet Physiol* 313: 587–596, 2010. doi:10.1002/jez.632.
26. **Chomczynski P, Sacchi N.** Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159, 1987. doi:10.1016/0003-2697(87)90021-2.
27. **Cottrill KA, Chan SY, Loscalzo J.** Hypoxamirs and mitochondrial metabolism. *Antioxid Redox Signal* 21: 1189–1201, 2014. doi:10.1089/ars.2013.5641.
28. **Dharap A, Vemuganti R.** Ischemic pre-conditioning alters cerebral microRNAs that are upstream to neuroprotective signaling pathways. *J Neurochem* 113: 1685–1691, 2010.
29. **Dietrich A, Wallet C, Iqbal RK, Gualberto JM, Lotfi F.** Organellar non-coding RNAs: emerging regulation mechanisms. *Biochimie* 117: 48–62, 2015. doi:10.1016/j.biochi.2015.06.027.
30. **Duerr JM, Podrabsky JE.** Mitochondrial physiology of diapausing and developing embryos of the annual killifish *Austrofundulus limnaeus*:

implications for extreme anoxia tolerance. *J Comp Physiol B* 180: 991–1003, 2010. doi:10.1007/s00360-010-0478-6.
31. **Eisen M, de Hoon M, Imoto S, Miyano S.** Cluster, in *C Clustering Library version 152*. Stanford, CA: Stanford University, 1998.
32. **Esteller M.** Non-coding RNAs in human disease. *Nat Rev Genet* 12: 861–874, 2011. doi:10.1038/nrg3074.
33. **Fergusson-Kolmes L, Podrabsky JE.** Differential effects of anoxia on heart rate in developmental stages of the annual killifish *Austrofundulus limnaeus* that differ in their tolerance of anoxia. *J Exp Zool A Ecol Genet Physiol* 307: 419–423, 2007. doi:10.1002/jez.395.
34. **Friedman RC, Farh KKH, Burge CB, Bartel DP.** Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 19: 92–105, 2009. doi:10.1101/gr.082701.108.
35. **Fu Y, Lee I, Lee YS, Bao X.** Small non-coding transfer RNA-derived RNA fragments (tRFs): Their biogenesis, function and implication in human diseases. *Genomics Inform* 13: 94–101, 2015. doi:10.5808/GI.2015.13.4.94.
36. **Garcia-Silva MR, Cabrera-Cabrera F, Güida MC, Cayota A.** Hints of tRNA-derived small RNAs role in RNA silencing mechanisms. *Genes (Basel)* 3: 603–614, 2012. doi:10.3390/genes3040603.
37. **Garcia-Silva MR, Cabrera-Cabrera F, Guida MC, Cayota A.** Novel aspects of tRNA-derived small RNAs with potential impact in infectious diseases. *Adv Biosci Biotechnol* 4: 17–25, 2013. doi:10.4236/abb.2013.45A002.
38. **Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J.** Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 5: R80, 2004. doi:10.1186/gb-2004-5-10-r80.
39. **Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ.** miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res* 34: D140–D144, 2006. doi:10.1093/nar/gkj112.
40. **Grimson A, Farh KK-H, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP.** MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell* 27: 91–105, 2007. doi:10.1016/j.molcel.2007.06.017.
41. **Gurtan AM, Sharp PA.** The role of miRNAs in regulating gene expression networks. *J Mol Biol* 425: 3582–3600, 2013. doi:10.1016/j.jmb.2013.03.007.
42. **Hadj-Moussa H, Moggridge JA, Luu BE, Quintero-Galvis JF, Gaitán-Espitia JD, Nespolo RF, Storey KB.** The hibernating South American marsupial, *Dromiciops gliroides*, displays torpor-sensitive microRNA expression patterns. *Sci Rep* 6: 24627, 2016. doi:10.1038/srep24627.
43. **Hagen JW, Lai EC.** microRNA control of cell-cell signaling during development and disease. *Cell Cycle* 7: 2327–2332, 2008. doi:10.4161/cc.6447.
44. **Hand SC, Denlinger DL, Podrabsky JE, Roy R.** Mechanisms of animal diapause: recent developments from nematodes, crustaceans, insects, and fish. *Am J Physiol Regul Integr Comp Physiol* 310: R1193–R1211, 2016. doi:10.1152/ajpregu.00250.2015.
45. **Hand SC, Hardewig I.** Downregulation of cellular metabolism during environmental stress: mechanisms and implications. *Annu Rev Physiol* 58: 539–563, 1996. doi:10.1146/annurev.ph.58.030196.002543.
46. **Hardewig I, Anchordoguy TJ, Crawford DL, Hand SC.** Profiles of nuclear and mitochondrial encoded mRNAs in developing and quiescent embryos of *Artemia franciscana*. *Mol Cell Biochem* 158: 139–147, 1996. doi:10.1007/BF00225840.
47. **Haussecker D, Huang Y, Lau A, Parameswaran P, Fire AZ, Kay MA.** Human tRNA-derived small RNAs in the global regulation of RNA silencing. *RNA* 16: 673–695, 2010. doi:10.1261/rna.2000810.
48. **He J, Jiang B-H.** Interplay between reactive oxygen species and microRNAs in cancer. *Curr Pharmacol Rep* 2: 82–90, 2016. doi:10.1007/s40495-016-0051-4.
49. **He L, Hannon GJ.** MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* 5: 522–531, 2004. doi:10.1038/nrg1379.
50. **Hrbek T, Taphorn DC, Thomerson JE.** Molecular phylogeny of *Austrofundulus* Myers (Cyprinodontiformes: Rivulidae), with revision of the genus and the description of four new species. *Zootaxa* 825: 1–39, 2005. doi:10.11646/zootaxa.825.1.1.
51. **Ishizu H, Siomi H, Siomi MC.** Biology of PIWI-interacting RNAs: new insights into biogenesis and function inside and outside of germlines. *Genes Dev* 26: 2361–2373, 2012. doi:10.1101/gad.203786.112.
52. **Keenan SW, Hill CA, Kandoth C, Buck LT, Warren DE.** Transcriptomic responses of the heart and brain to anoxia in the Western Painted Turtle. *PLoS One* 10: e0131669, 2015. doi:10.1371/journal.pone.0131669.
53. **Krützfeldt J, Stoffel M.** MicroRNAs: a new class of regulatory genes affecting metabolism. *Cell Metab* 4: 9–12, 2006. doi:10.1016/j.cmet.2006.05.009.
54. **Kulshreshtha R, Davuluri RV, Calin GA, Ivan M.** A microRNA component of the hypoxic response. *Cell Death Differ* 15: 667–671, 2008. doi:10.1038/sj.cdd.4402310.
55. **Kulshreshtha R, Ferracin M, Negrini M, Calin GA, Davuluri RV, Ivan M.** Regulation of microRNA expression: the hypoxic component. *Cell Cycle* 6: 1426–1431, 2007. doi:10.4161/cc.6.12.4410.
56. **Kulshreshtha R, Ferracin M, Wojcik SE, Garzon R, Alder H, Agosto-Perez FJ, Davuluri R, Liu C-G, Croce CM, Negrini M, Calin GA, Ivan M.** A microRNA signature of hypoxia. *Mol Cell Biol* 27: 1859–1867, 2007. doi:10.1128/MCB.01395-06.
57. **Land SC, Buck LT, Hochachka PW.** Response of protein synthesis to anoxia and recovery in anoxia-tolerant hepatocytes. *Am J Physiol Regul Integr Comp Physiol* 265: R41–R48, 1993.
58. **Lang-Ouellette D, Morin P Jr.** Differential expression of miRNAs with metabolic implications in hibernating thirteen-lined ground squirrels, *Ictidomys tridecemlineatus*. *Mol Cell Biochem* 394: 291–298, 2014. doi:10.1007/s11010-014-2105-4.
59. **Latronico MV, Condorelli G.** The might of microRNA in mitochondria. *Circ Res* 110: 1540–1542, 2012. doi:10.1161/CIRCRESAHA.112.271312.
60. **Lee S-T, Chu K, Jung K-H, Yoon H-J, Jeon D, Kang K-M, Park K-H, Bae E-K, Kim M, Lee SK, Roh J-K.** MicroRNAs induced during ischemic preconditioning. *Stroke* 41: 1646–1651, 2010. doi:10.1161/STROKEAHA.110.579649.
61. **Lee YS, Shibata Y, Malhotra A, Dutta A.** A novel class of small RNAs: tRNA-derived RNA fragments (tRFs). *Genes Dev* 23: 2639–2649, 2009. doi:10.1101/gad.1837609.
62. **Love MI, Huber W, Anders S.** Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15: 550, 2014. doi:10.1186/s13059-014-0550-8.
63. **Lund AH.** miR-10 in development and cancer. *Cell Death Differ* 17: 209–214, 2010. doi:10.1038/cdd.2009.58.
64. **Luu BE, Storey KB.** Dehydration triggers differential microRNA expression in *Xenopus laevis* brain. *Gene* 573: 64–69, 2015. doi:10.1016/j.gene.2015.07.027.
65. **Lyons PJ, Crapoulet N, Storey KB, Morin P Jr.** Identification and profiling of miRNAs in the freeze-avoiding gall moth E*piblema scudderiana* via next-generation sequencing. *Mol Cell Biochem* 410: 155–163, 2015. doi:10.1007/s11010-015-2547-3.
66. **Lyons PJ, Govaere L, Crapoulet N, Storey KB, Morin PJ.** Characterization of cold-associated microRNAs in the freeze-tolerant gall fly *Eurosta solidaginis* using high-throughput sequencing. *Comp Biochem Physiol Part D Genomics Proteomics* 20: 95–100, 2016. doi:10.1016/j.cbd.2016.08.007.
67. **Meller CL, Meller R, Simon RP, Culpepper KM, Podrabsky JE.** Cell cycle arrest associated with anoxia-induced quiescence, anoxic preconditioning, and embryonic diapause in embryos of the annual killifish *Austrofundulus limnaeus*. *J Comp Physiol B* 182: 909–920, 2012. doi:10.1007/s00360-012-0672-9.
68. **Meller CL, Meller R, Simons RP, Podrabsky JE.** Patterns of ubiquitylation and SUMOylation associated with exposure to anoxia in embryos of the annual killifish *Austrofundulus limnaeus*. *J Comp Physiol B* 184: 235–247, 2014. doi:10.1007/s00360-013-0791-y.
69. **Mogilyansky E, Rigoutsos I.** The miR-17/92 cluster: a comprehensive update on its genomics, genetics, functions and increasingly important and numerous roles in health and disease. *Cell Death Differ* 20: 1603–1614, 2013. doi:10.1038/cdd.2013.125.
70. **Nallamshetty S, Chan SY, Loscalzo J.** Hypoxia: a master regulator of microRNA biogenesis and activity. *Free Radic Biol Med* 64: 20–30, 2013. doi:10.1016/j.freeradbiomed.2013.05.022.
71. **Nawrocki EP, Burge SW, Bateman A, Daub J, Eberhardt RY, Eddy SR, Floden EW, Gardner PP, Jones TA, Tate J, Finn RD.** Rfam 12.0: updates to the RNA families database. *Nucleic Acids Res* 43, D1: D130–D137, 2015. doi:10.1093/nar/gku1063.

72. **Nilsson GE, Lutz PL.** Anoxia tolerant brains. *J Cereb Blood Flow Metab* 24: 475–486, 2004. doi:10.1097/00004647-200405000-00001.
73. **Podrabsky J, Riggs C, Wagner J.** Tolerance of Environmental Stress, in *Annual Fishes Life History Strategy, Diversity, and Evolution* (Berois N, García G, De Sá R, editors). Boca Raton, FL: CRC Press, Taylor & Francis, 2016, p. 159–184.
74. **Podrabsky J, Romney A, Culpepper K.** Alternative Developmental Pathways, in *Annual Fishes Life History Strategy, Diversity, and Evolution* (Berois N, García G, De Sá R, editors). Boca Raton, FL: CRC Press, Taylor & Francis, 2016, p. 63–73.
75. **Podrabsky JE.** Husbandry of the annual killifish *Austrofundulus limnaeus* with special emphasis on the collection and rearing of embryos. *Environ Biol Fishes* 54: 421–431, 1999. doi:10.1023/A:1007598320759.
76. **Podrabsky JE, Carpenter JF, Hand SC.** Survival of water stress in annual fish embryos: dehydration avoidance and egg envelope amyloid fibers. *Am J Physiol Regul Integr Comp Physiol* 280: R123–R131, 2001.
77. **Podrabsky JE, Hand SC.** The bioenergetics of embryonic diapause in an annual killifish, *Austrofundulus limnaeus*. *J Exp Biol* 202: 2567–2580, 1999.
78. **Podrabsky JE, Hand SC.** Depression of protein synthesis during diapause in embryos of the annual killifish *Austrofundulus limnaeus*. *Physiol Biochem Zool* 73: 799–808, 2000. doi:10.1086/318106.
79. **Podrabsky JE, Hrbek T, Hand SC.** Physical and chemical characteristics of ephemeral pond habitats in the Maracaibo basin and Llanos region of Venezuela. *Hydrobiologia* 362: 67–77, 1997. doi:10.1023/A:1003168704178.
80. **Podrabsky JE, Lopez JP, Fan TWM, Higashi R, Somero GN.** Extreme anoxia tolerance in embryos of the annual killifish *Austrofundulus limnaeus*: insights from a metabolomics analysis. *J Exp Biol* 210: 2253–2266, 2007. doi:10.1242/jeb.005116.
81. **Podrabsky JE, Menze MA, Hand SC.** Long-Term survival of anoxia despite rapid ATP decline in embryos of the annual killifish Austrofundulus limnaeus. *J Exp Zool A Ecol Genet Physiol* 317: 524–532, 2012. doi:10.1002/jez.1744.
82. **Podrabsky JE, Riggs CL, Duerr JM.** Anoxia tolerance during vertebrate development - insights from studies on the annual killifish *Austrofundulus limnaeus*, in *Anoxia*, edited by Padilla P. Rijeka, Croatia: InTech, 2012, p. 3–24.
83. **R Core Team.** *A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing, 2016.
84. **Ro S, Ma H-Y, Park C, Ortogero N, Song R, Hennig GW, Zheng H, Lin Y-M, Moro L, Hsieh J-T, Yan W.** The mitochondrial genome encodes abundant small noncoding RNAs. *Cell Res* 23: 759–774, 2013. doi:10.1038/cr.2013.37.
85. **Röther S, Meister G.** Small RNAs derived from longer non-coding RNAs. *Biochimie* 93: 1905–1915, 2011. doi:10.1016/j.biochi.2011.07.032.
86. **Rüegger S, Großhans H.** MicroRNA turnover: when, how, and why. *Trends Biochem Sci* 37: 436–446, 2012. doi:10.1016/j.tibs.2012.07.002.
87. **Saikia M, Hatzoglou M.** The many virtues of tRNA-derived stress-induced RNAs (tiRNAs): Discovering novel mechanisms of stress response and effect on human health. *J Biol Chem* 290: 29761–29768, 2015. doi:10.1074/jbc.R115.694661.
88. **Saikia M, Krokowski D, Guan B-J, Ivanov P, Parisien M, Hu GF, Anderson P, Pan T, Hatzoglou M.** Genome-wide identification and quantitative analysis of cleaved tRNA fragments induced by cellular stress. *J Biol Chem* 287: 42708–42725, 2012. doi:10.1074/jbc.M112.371799.
89. **Saldanha A.** *Java TreeView*. Source Forage, 2003.
90. **Schieber M, Chandel NS.** ROS function in redox signaling and oxidative stress. *Curr Biol* 24: R453–R462, 2014. doi:10.1016/j.cub.2014.03.034.
91. **Slagsvold KH, Rognmo O, Høydal M, Wisløff U, Wahba A.** Remote ischemic preconditioning preserves mitochondrial function and influences myocardial microRNA expression in atrial myocardium during coronary bypass surgery. *Circ Res* 114: 851–859, 2014. doi:10.1161/CIRCRESAHA.114.302751.
92. **Solaini G, Sgarbi G, Baracca A.** Oxidative phosphorylation in cancer cells. *Biochim Biophys Acta* 1807: 534–542, 2011. doi:10.1016/j.bbabio.2010.09.003.
93. **Song JL, Nigam P, Tektas SS, Selva E.** microRNA regulation of Wnt signaling pathways in development and disease. *Cell Signal* 27: 1380–1391, 2015. doi:10.1016/j.cellsig.2015.03.018.
94. **Srinivasan H, Das S.** Mitochondrial miRNA (MitomiR): a new player in cardiovascular health. *Can J Physiol Pharmacol* 93: 855–861, 2015. doi:10.1139/cjpp-2014-0500.
95. **Storey KB, Biggar KK.** Exploration of low temperature microRNA function in an anoxia tolerant vertebrate ectotherm, the red eared slider turtle (*Trachemys scripta elegans*). *J Therm Biol*: 68A: 139–146, 2017. doi:10.1016/j.jtherbio.2016.09.008.
96. **Thatcher EJ, Flynt AS, Li N, Patton JR, Patton JG.** MiRNA expression analysis during normal zebrafish development and following inhibition of the Hedgehog and Notch signaling pathways. *Dev Dyn* 236: 2172–2180, 2007. doi:10.1002/dvdy.21211.
97. **Wagner JT, Herrejon Chavez F, Podrabsky JE.** Mitochondrial DNA sequence and lack of response to anoxia in the annual killifish *Austrofundulus limnaeus*. *Front Physiol* 7: 379, 2016. doi:10.3389/fphys.2016.00379.
98. **Wagner JT, Podrabsky JE.** Gene expression patterns that support novel developmental stress buffering in embryos of the annual killifish *Austrofundulus limnaeus*. *Evodevo* 6: 2, 2015. doi:10.1186/2041-9139-6-2.
99. **Wang Y, Li H, Sun Q, Yao Y.** Characterization of small RNAs derived from tRNAs, rRNAs and snoRNAs and their response to heat stress in wheat seedlings. *PLoS One* 11: e0150933, 2016. doi:10.1371/journal.pone.0150933.
100. **Weick E-M, Miska EA.** piRNAs: from biogenesis to function. *Development* 141: 3458–3471, 2014. doi:10.1242/dev.094037.
101. **Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, de Bruijn E, Horvitz HR, Kauppinen S, Plasterk RHA.** MicroRNA expression in zebrafish embryonic development. *Science* 309: 310–311, 2005. doi:10.1126/science.1114519.
102. **Wourms JP.** Developmental biology of annual fishes. I. Stages in the normal development of Austrofundulus myersi Dahl. *J Exp Zool* 182: 143–167, 1972. doi:10.1002/jez.1401820202.
103. **Wourms JP.** The developmental biology of annual fishes. II. Naturally occurring dispersion and reaggregation of blastomers during the development of annual fish eggs. *J Exp Zool* 182: 169–200, 1972. doi:10.1002/jez.1401820203.
104. **Wourms JP.** The developmental biology of annual fishes. 3. Pre-embryonic and embryonic diapause of variable duration in the eggs of annual fishes. *J Exp Zool* 182: 389–414, 1972. doi:10.1002/jez.1401820310.
105. **Wu C-W, Biggar KK, Storey KB.** Dehydration mediated microRNA response in the African clawed frog *Xenopus laevis*. *Gene* 529: 269–275, 2013. doi:10.1016/j.gene.2013.07.064.
106. **Wu C-W, Biggar KK, Storey KB.** Expression profiling and structural characterization of microRNAs in adipose tissues of hibernating ground squirrels. *Genomics Proteomics Bioinformatics* 12: 284–291, 2014. doi:10.1016/j.gpb.2014.08.003.
107. **Wu L, Fan J, Belasco JG.** MicroRNAs direct rapid deadenylation of mRNA. *Proc Natl Acad Sci USA* 103: 4034–4039, 2006. doi:10.1073/pnas.0510928103.
108. **Ye Y, Perez-Polo JR, Qian J, Birnbaum Y.** The role of microRNA in modulating myocardial ischemia-reperfusion injury. *Physiol Genomics* 43: 534–542, 2011. doi:10.1152/physiolgenomics.00130.2010.
109. **Yuan L, Geiser F, Lin B, Sun H, Chen J, Zhang S.** Down but not out: the role of microRNAs in hibernating bats. *PLoS One* 10: e0135064, 2015. doi:10.1371/journal.pone.0135064.