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Small Noncoding RNA Expression During Extreme Anoxia Tolerance of Annual Killifish (*Austrofundulus limnaeus*) Embryos

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AUTHOR CONTRIBUTIONS

CR and JP designed the experiment and determined the sncRNA processing parameters. CR conducted the experiments and analyzed the data. The manuscript was written by CR and edited by JP. Both authors agree on the final manuscript.

RUNNING HEAD

sncRNA in Anoxia-Tolerant Annual Killifish Embryos

KEYWORDS

miRNA, annual killifish, diapause, preconditioning

ABSTRACT

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 4 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.

INTRODUCTION

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 up-regulation 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 non-coding 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) allows for quick regulation of mRNA stability and

translation. Additionally, miRNAs are found and highly conserved throughout the animal kingdom (9).

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 (non-lethal 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

114 necessary to extend survival of anoxia, not just to immediately respond to the lack of
115 oxygen.

116 While most studies analyzing sncRNA expression in response to hypoxia and
117 hypometabolism focus on miRNAs, other classes of sncRNAs are also emerging as
118 important players in the regulation of cellular and organismal function. SncRNAs are
119 also derived from tRNA, rRNA, long non-coding RNA, small nucleolar RNA, and piwi-
120 RNA; many of these have gene regulatory functions (32, 35, 36, 51, 85, 99, 100).
121 Environmental stressors, such as dehydration and oxidative stress, generate tRNA-
122 derived fragments in diverse organisms, ranging from yeast to humans (36, 37, 47, 61,
123 87, 88). Classes of mitochondria-associated sncRNAs have also come into view. A new
124 class of miRNAs, called mitomiRs, localize to the mitochondria, either by translocation
125 or physical association (5, 6, 8, 59, 94). The mitochondrial genome also produces
126 sncRNAs, termed mitosRNAs (84). Many sncRNAs have miRNA-like gene regulatory
127 properties (32), however discovery of new functions and mechanisms continues. Recent
128 advances in understanding the roles of miRNA in modulation of gene expression,
129 particularly during metabolic depression and anoxia, hypoxia, or ischemia, make
130 sncRNAs a promising avenue of investigation of anoxia tolerance mechanisms in *A.*
131 *limnaeus* embryos.

132 The ephemeral pond habitat of *A. limnaeus* imposes extreme environmental
133 conditions, including anoxia, on developing embryos (50, 79). As a result, extreme
134 anoxia tolerance is critical to the persistence of the species. Three periods of metabolic
135 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 (Table 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 a week without oxygen (80, 82) and by 20 dpd, shortly prior to hatching, embryos survive less than 24 h of anoxia (Table 1). Additionally, anoxic preconditioning (82), a brief non-lethal exposure to anoxia, extends the anoxia tolerance of 8-12 dpd stage embryos, as previously described in mammals. Preconditioning, 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

158 exposure to anoxia and recovery. Dormant D2 embryos display a unique sncRNA
159 profile under normoxia and in response to anoxia, relative to the actively developing
160 and metabolically active post-D2 embryos whose profiles are more similar to each
161 other. Known stress- and hypoxia-responsive miRNAs are identified in the *A. limnaeus*
162 dataset. Additionally, many novel sncRNAs are also identified, including highly
163 anoxia-responsive sequences derived from the mitochondrial genome.

METHODS

Embryo rearing and staging

Embryos of *Austrofundulus limnaeus* were collected from adult spawning pairs and cared for according to established husbandry methods (75) in accordance with approved Portland State University IACUC protocols (PSU IACUC protocol #33). At 25˚C embryos normally enter D2 at 24 days post-fertilization (dpf). In order 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 using established methods (75). Embryos were staged according to Wourms' staging (WS)(102-104).

Experimental design

Details of the experimental design and the physiological phenotypes of the developmental stages used in this study are provided in Table 1. Embryos were exposed to anoxia and aerobic recovery following anoxia, and sampled for sncRNA expression profiling. Diapause 2, 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. 20 dpd recovery embryos were sampled at 2

and 24 h of recovery. For each stage, four biological replicates (n=4), comprised of 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 pre-weighed 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 using TRIzol™ reagent (Invitrogen Inc., Carlsbad, California), as previously described (26, 98). Briefly, samples were homogenized in TRIzol, phase separated with chloroform, and RNA was precipitated

from the aqueous phase by incubating overnight 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, USA). 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 (Table S1). 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 using 1 µg total RNA as input for the TruSeq® Small RNA sequencing kit (Illumina, Inc. 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 (OHSU MPSSR). Library quality was assessed prior to sequencing using a DNA-1000 chip on a model 2100 Bioanalyzer (Agilent Technologies). Real time quantitative PCR was used to quantify libraries prior to 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 (Table S1). All cycle lengths captured the small RNAs and part of the 3′ adapter sequence. 12 samples were multiplexed per flow cell lane. Unique indices 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 (SRA). Accession numbers are listed in 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 prior to and following trimming. Sequences were mapped to the *Austrofundulus limnaeus* genome

(Austrofundulus_limnaeus-1.0 GCF_001266775.1 and the *Austrofundulus 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), in order 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 2 mismatches, and 2 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 using the likelihood ratio test (LRT) 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

272 expression values for analysis of differential expression in response to anoxia and
273 recovery from anoxia within a stage.
274 Highly differentially expressed sncRNAs of interest were clustered by expression
275 pattern with Cluster 3.0 (31) for heat map generation. Data were log transformed and
276 then organized by genes into clusters, based on k-means, using the Euclidean distance
277 similarity metric for 100 runs. Clustered genes were viewed as a heat-map using Java
278 TreeView (89).
279
280 *Literature Search for miRNAs*
281 To compare microRNAs identified in *A. limnaeus* to known stress,
282 hypometabolism, ischemia, hypoxia, and ischemic-preconditioning (IP)-responsive
283 miRNAs in the literature we created a database of miRNAs known to respond to these
284 conditions (Table S2). The list of stress and hypometabolism-responsive miRNAs
285 included all examples of miRNAs differentially expressed in response to
286 hypometabolism found by searching "miRNA hypometabolism" in google scholar.
287 Articles directly pertaining to hypoxia (ie in the title) were skipped. For the hypoxia-
288 responsive miRNA list, we referenced the latest most comprehensive review on the
289 topic by Kulshreshtha et al. 2008 (54) and supplemented it with a specific recent review
290 on miRNAs in myocardial infarction (18), reviews on hypoxamirs (4, 27), and recent
291 reviews and primary articles on miRNAs in preconditioning (28, 91, 108). MiRNA
292 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 & 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 Table S3. Below, the potential importance of both constitutively expressed sncRNAs and anoxia-induced sncRNAs that may support anoxia tolerance in *A. limnaeus* are discussed. 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. 1A), but is not altered by exposure to anoxia (Fig. 1B). 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

316 comparable coverage (Table 2, Table S1). Additionally, the number of unique small
317 RNA reads in the catalog does not differ significantly by stage or by treatment (data not
318 shown), justifying further comparison of the sncRNA expression profiles between
319 stages and treatments.
320
321 *Stress-responsive miRNAs are highly expressed in all developmental stages*
322 At each embryonic stage, many of the top 100 most abundant sncRNAs annotate
323 to known stress-responsive miRNAs. Many of the sequences enriched in 4 dpd
324 embryos, relative to D2 embryos, are also abundant in 12 dpd embryos (Fig. 2A clusters
325 1 & 4). 68 of the 100 most highly expressed sequences in 4 dpd embryos are also highly
326 expressed in 12 dpd and/or 20 dpd embryos (Fig. 2B). Of these sequences, ~ 63% (43 of
327 68) annotate to known stress-responsive miRNAs (Table S4). Dormant (D2) embryos
328 have a unique sncRNA transcriptome (see below), and only share 18 of their most
329 abundant sncRNA sequences with post-D2 stages (Fig. 2B, Table 3). miRNAs with
330 known stress-responsive function account for nearly all of these shared sequences. This
331 result suggests that baseline expression of stress-responsive sncRNAs may predispose
332 *A. limnaeus* embryos to tolerate stress, given that D2 embryos are the most stress-
333 tolerant developmental stage of *A. limnaeus* (73). Despite enrichment of known
334 sequences documented to respond to stress, these miRNAs may also have different
335 roles. A single miRNA can target multiple genes and consequently have many
336 biological functions. Several variants of miR-10b and miR-92a are abundant in each
337 embryonic stage (Table 3), and these sequences are often dysregulated in cancer but

338 also have normal functions in development (63, 69). Dissecting the function of such
339 miRNAs in a general stress-response in *A. limnaeus* requires detailed experiments,
340 including the identification of mRNA targets.
341
342 *Stage- and tolerance-specific patterns of sncRNA abundance*
343 Each embryonic stage, unique in its anoxia tolerance and physiology (Table 1),
344 displays a distinct sncRNA profile under normoxic conditions (Fig. 2, Table S4, Table
345 S5, Fig S2) and in response to anoxia (Fig. 3, Table S6, Fig. S2). Evaluation of these
346 expression patterns and the known differences in physiology, anoxia-tolerance, and
347 metabolic profiles of the stages allows for the identification of sequences that may be
348 adaptive for surviving anoxia and should be targeted for detailed study in the future.
349
350 *Diapause 2 embryos are uniquely poised for anoxic survival*
351 Dormant D2 embryos express unique sncRNA patterns compared to the actively
352 developing post-D2 stages. Expression of many D2-specific sequences (Fig. 2A clusters
353 2 & 3) prior to exposure to anoxia fits with the unique physiological state (profound
354 metabolic and developmental arrest) of D2 embryos (77). These D2-specific sequences
355 may support regulation of metabolism, protein synthesis, and cell cycle arrest
356 associated with entrance into D2 (44, 73, 74). They may also play a role in support of
357 anoxia tolerance as D2 embryos appear prepared for anaerobic metabolism even under
358 aerobic conditions, and thus few adjustments are needed during their entry into anoxia
359 (25, 30, 77).

D2 embryos may be prepared at baseline with a high abundance of sequences required to regulate the stressful 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. 3 clusters 4-1, 4-3, 4-5). In fact, 96% of the sncRNAs in Figure 3 cluster 4-1 are abundant at t=0 in D2 embryos (Fig. 2 clusters 1 & 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.

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. 4). 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)(Table 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. 3, groups B & 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. 3 cluster D2-2, Fig. 5B,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. 3 cluster D2-1, Fig. 5A). 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 (Fig. 3, Fig. 4), 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. 3 clusters 4-1, 4-3, 4-

404 5). These are the same clusters referenced above that are constitutively expressed in D2
405 embryos, but of low abundance or not expressed in normoxic post-D2 stages (Fig.
406 5A,B,C). Thus, it seems that highly anoxia-tolerant and metabolically active (4 dpd)
407 embryos must produce sncRNA sequences *de novo* during recovery, which highly
408 anoxia-tolerant and dormant embryos (D2) express in abundance regardless of
409 exposure to anoxia. Based on this expression we hypothesize that these sequences
410 support homeostasis during the critical transition from anoxia to normoxia in 4 dpd
411 embryos.

412 Dramatic changes in sncRNA abundance during recovery is not surprising given
413 that successful transition between anoxia and normoxia is essential for survival, but
414 particularly challenging from a cell biological perspective (11). Reperfusion-injury
415 occurs when restoration of circulation delivers a surge of oxygen to tissues and
416 increases the chances of reactive oxygen species (ROS) production (19). ROS damages
417 DNA, cell membranes, and proteins (90), and therefore threatens survival. Recent
418 discovery of miRNAs that regulate ROS production (48) and sense redox changes
419 (redoximiRs) (24), supports the idea that these recovery-responsive miRNAs could be
420 involved in preventing or ameliorating reperfusion injury. In 4 dpd embryos exposed to
421 anoxia, 17 miRNAs previously reported to play a role in regulating and responding to
422 ROS (Table 4) increase significantly in abundance during recovery, relative to normoxic
423 (t=0) levels (Fig 3. clusters 4-1, 4-3, 4-5). Therefore, we hypothesize that the recovery-
424 responsive miRNAs overwhelming the sncRNA response to anoxia in 4 dpd embryos
425 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 4 dpd embryos survive anoxia for about the same about of time (Table 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). Further, 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 dpd and 20 dpd).

The 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. 3 cluster 4-2). These sequences initially decrease in abundance after 4 h of anoxia, compared to baseline levels, but drastically increase in abundance by 24 h of anoxia (Fig. 5G,H,I). The same sequences display similar expression patterns in 12 dpd embryos, but for the most part only in 4 dpd

448 embryos does the change in abundance rise to the level of statistical significance. In
449 contrast to the above discussion, these sequences are not abundant during recovery.
450 This pattern suggests possible degradation or consumption of the sequences during
451 transitions into and out of anoxia. The few published studies on sncRNA expression
452 during anoxia that include time-course analyses generally show a graded increase or
453 decrease in expression as the anoxic bout continues (15, 95). Synthesis of these
454 sequences during anoxia is surprising since the lack of oxygen adversely affects the
455 canonical miRNA biogenesis pathway (70). However, most of these sequences are not
456 similar to any known miRNAs, but rather map to the mitochondrial genome of *A.*
457 *limnaeus.* As non-canonical miRNAs, their generation may be possible under anoxia.
458 The 4 dpd anoxia-responsive cluster (Fig. 3 cluster 4-2) is particularly enriched for
459 sequences derived from mitochondrial transfer-RNAs (tRNAs) (Fig. 6). While a few
460 mitochondria-derived sequences increase in abundance during recovery from anoxia
461 (Fig. 3 clusters 4-3 & 4-5), mitochondria-derived sequences comprise the majority (75%)
462 of sncRNAs that increase in abundance during anoxia in 4 dpd embryos. This unique
463 signature of anoxia-responsive mitochondria-derived sncRNAs characterizes 4 dpd
464 embryos, the most anoxia-tolerant and metabolically active stage. We hypothesize that
465 these mitochondria-derived sncRNAs may be central to the development of extreme
466 anoxia-tolerance in active *A. limnaeus* embryos.

467 Mitochondria-derived small RNAs (mitosRNAs) (84) and mitomiRs (miRNAs
468 encoded in the nuclear genome and localized to mitochondria) (8, 29, 94) have recently
469 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. Further, 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%(Table 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 is comprised of sequences that dramatically increase in expression at 2 and 24 h of recovery (Figs. 3 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. 5J,K,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

514 stress/hypometabolism-responsive sequences (Table 5). Of these sequences, over half
515 (54%) annotate to known preconditioning-responsive miRNAs, but only one of the
516 sequences (mir-153) is solely documented as preconditioning-responsive in the
517 literature. The majority of these putative preconditioning-responsive sequences respond
518 to either stress/hypometabolism or a variety of stresses including hypoxia, and
519 preconditioning. This overlap led us to hypothesize that preconditioning may activate a
520 robust conserved general stress response in *A. limnaeus* embryos that supports survival
521 of subsequent exposures to anoxia. Hypoxia, ischemia and preconditioning responsive
522 sncRNAs from mammalian species are not represented in large numbers in these
523 sequences, indicating that the preconditioning response in *A. limnaeus*, a highly anoxia-
524 tolerant species, may be distinct from that of anoxia-sensitive mammals.
525
526 *Anoxia-sensitive 20 dpd embryos do not mount a robust sncRNA response to anoxia*
527 Metabolically active embryos near hatching and sensitive to anoxia (20 dpd,
528 Table 1), fail to mount a robust sncRNA response to anoxia. Exposure to anoxia only
529 induces changes in expression of 64 sequences in these embryos, compared with 224
530 and 772 sequences in 12 dpd and 4 dpd embryos, respectively (Fig. 3, Fig. 4). Of the few
531 sequences that change in abundance about half are shared with those differentially
532 expressed in 12 dpd embryos (Fig. 3 clusters 20-1 & 20-2). In general, these sequences
533 are less abundant in 20 dpd embryos than in other post-D2 embryos, indicating a muted
534 response (Fig. 5M,N,O). A diminished sncRNA response to anoxia in 20 dpd embryos,
535 relative to other post-D2 embryos, may underlie the reduced anoxia tolerance and

limited ability to suppress metabolism observed in these pre-hatching embryos. In contrast to the anoxia-tolerant 4 dpd embryos, the heart continues to beat during anoxia in embryos 12 dpd and older (33; JEP, 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. 3 cluster 20-2 & 20-3). First, sequences abundant under normoxia decline in abundance and do not regain normoxic levels within 24 h of recovery (Fig. 3 cluster 20-2). Of the 7 sequences with this distinct pattern, 4 have not previously been described and 3 annotate to variants of mir-1b. Mir-1 variants are associated with ischemic-preconditioning (91) and hypometabolism (22, 42) (Table S2). Recovering from anoxia produces an increase in transcript abundance of a second group of sncRNAs in 20 dpd embryos (Fig. 3 cluster 20-3). Many of these sequences are also recovery-responsive in extremely anoxia-tolerant 4 dpd embryos (Fig. 5O). The significance of these expression patterns remains unclear, but the generally reduced response in anoxia-sensitive 20 dpd embryos compared to 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. In order 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 under-expression 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.

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The authors have no conflicts of interest to disclose.

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886 **Table 1.** Anoxia tolerance, physiology, and sampling of *Austrofundulus limnaeus*
887 developmental stages included in this study

WS[1]	dpd[2]	LT_{50}, days[3]	AP[4]	Sampling (hrs)[5]	Metabolic Depression[6]	Heart Rate[7]	Morphology	
32	0	62	Un-known	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	

888 1. WS = Wourms' stage (102)
889 2. dpd = days post-diapause II
890 3. Time to 50% mortality in anoxia at 25°C for 0, 4, and 12 dpd embryos(1) (82, 80). Data
891 for 20 dpd embryos is first presented here.
892 4. AP = Percent change in survival following anoxic preconditioning (82). Data for 20
893 dpd embryos is first presented here.
894 5. A = anoxia and R = recovery
895 6. Type of metabolic depression involved
896 7. Heart rate in beats/min during normoxic incubation at 25°C (3)
897
898

899 **Table 2.** Total number of reads for each developmental stage that contribute to the
900 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

901
902

903 **Table 3.** Sequences shared with D2 embryos that are present in the top 100 most
904 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

905 S = stress responsive; H = hypoxia responsive; IP = ischemic preconditioning responsive
906
907

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

Sequence	*Annotation*	*Function documented in literature*	*Cluster*[1]
AGGATATCATCTTATACTGTAA	mir-144	regulate generation of ROS[2]	4-5
AGGCGGAGACTTGAGCAATT	mir-25	regulate generation of ROS[2]	4-5
AGGCGGAGACTTGAGCAATTG	mir-25	regulate generation of ROS[2]	4-5
ATCACATTGCCAGGGATTA	mir-23b	regulate generation of ROS[2]	4-1
ATCACATTGCCAGGGATTAC	mir-23b	regulate generation of ROS[2]	4-5
ATTGCACTTGTCTCGGTCT	mir-25	regulate generation of ROS[2]	4-5
CATTGCACTTGTCTCGG	mir-25	regulate generation of ROS[2]	4-5
CATTGCACTTGTCTCGGT	mir-25	regulate generation of ROS[2]	4-1
CATTGCACTTGTCTCGGTC	mir-25	regulate generation of ROS[2]	4-5
CATTGCACTTGTCTCGGTCT	mir-25	regulate generation of ROS[2]	4-5
GGATATCATCTTATACTGTAA	mir-144	regulate generation of ROS[2]	4-5
TAACACTGTCTGGTAACGA	mir-200a	affect ROS levels in cancer cells[3]	4-5
TAACACTGTCTGGTAACGAT	mir-200a	affect ROS levels in cancer cells[3]	4-5
TAGGTAGTTTCATGTTGTTG	mir-196	regulate Nrf2 and related regulatory proteins[2]	4-3
TGAGAACTGAATTCCATAGAT	mir-146a	regulate generation of ROS[2]	4-3
TTAATGCTAATCGTGATAGGG	mir-155	regulate Nrf2 and related regulatory proteins[2]	4-5
TTGCATAGTCACAAAAGTGA	mir-153	regulate Nrf2 and related regulatory proteins[2]	4-3

910 1. Differential expression cluster as presented in Figure 3.
911 2. Cheng et al. 2013 (24)
912 3. He and Jiang 2016 (48)
913

914 **Table 5.** Preconditioning-responsive sncRNAs annotate to known stress-responsive
915 miRNAs in 12 dpd embryos.

miRNA class	**A. limnaeus** ***Sequence***	***Annotation***	***Function in literature***[1]	***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 5K)	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 (1)	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 5J)	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)

916 1. See Supplemental Table 1 for more information.
917

FIGURE CAPTIONS

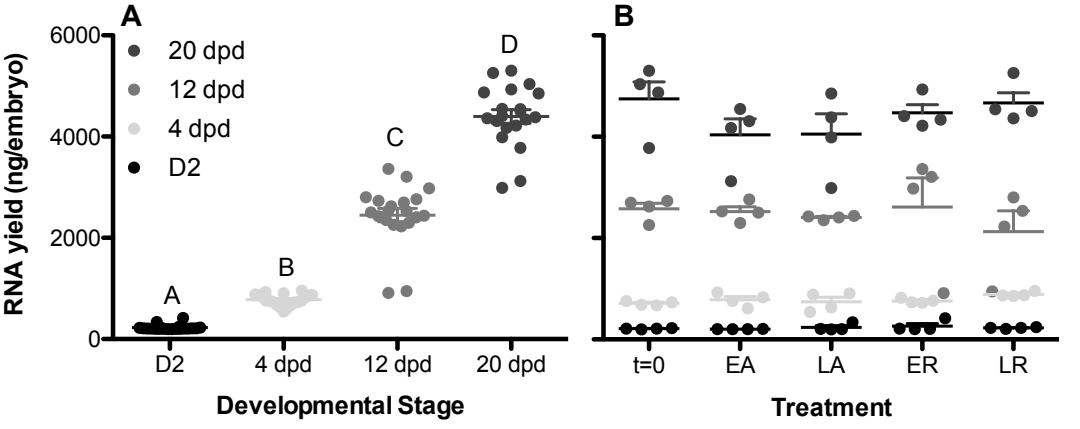
Figure 1. RNA yield per embryo in the four developmental stages investigated. (**A**) The total RNA extracted per embryo increases during development, likely due to cell proliferation and differentiation (ANOVA, $p < 0.0001$; Tukey's post hoc, $p < 0.05$; stages with different letters are statistically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

Figure 2. 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. Grey indicates a missing value due to the absence of that sncRNA in that developmental stage. Expression patterns were parsed into 5 basic clusters using K-means clustering. See Supplemental Table 5 and Supplemental Figure 1 for detail on these sequences. (**B**) Venn diagram of top 100 most abundant sequences expressed in normoxic embryos (t = 0). See Table 3 for details on these abundant sequences

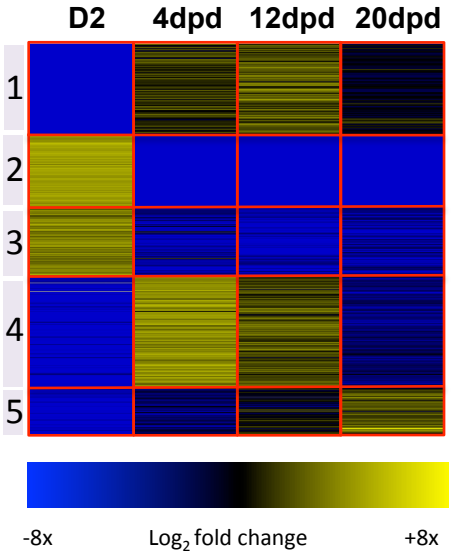
941 that are shared in D2 and post-D2 embryos and Supplemental Table 4 for details
942 on all the top 100 sequences.
943
944 **Figure 3.** Heat map of sncRNA differential expression in response to anoxia and
945 recovery from anoxia in each developmental stage. Highly differentially
946 expressed sncRNAs (adjusted p-value < 0.01, log_2fold change > 2, and
947 normalized mean expression across all samples > 25) were clustered within each
948 stage (cluster numbers are listed on the left side of the heat map). Within each
949 cluster, expression patterns are displayed for all stages, even though expression
950 may not be statistically different in the other stages. Because differential
951 expression was determined within each stage, some sncRNA sequences may be
952 represented in multiple stages. Within each stage, expression on the heat map
953 corresponds with exposure to anoxia and recovery from anoxia (indicated by the
954 gradient filled triangles above the heatmap; from left to right: t = 0 (normoxia),
955 early anoxia, late anoxia, early recovery, late recovery. For details on sampling
956 see Table 1. Log_2 fold change values were calculated relative to the mean
957 expression of all sncRNAs over all 80 samples (all replicates of all stages and
958 treatments). Yellow indicates increased expression relative to the mean while
959 blue indicates decreased expression. Grey indicates a missing value due to
960 absence of that sequence in that experimental treatment. Letters to the right of
961 the heat map indicate sequences that are represented in line graphs in Figure 5.

962 See Supplemental Table 6 and Supplemental Figure 2 for detail on these and
963 other differentially expressed sequences.
964
965 **Figure 4.** The relationship between anoxia tolerance and the number of highly
966 differentially expressed sequences for each stage. Note that D2 embryos have a
967 low number of differentially expressed sncRNAs, even though they have a high
968 tolerance of anoxia.
969
970 **Figure 5.** Normalized expression of the most significantly differentially
971 expressed (based on p-value) sequences in each developmental stage (see Figure
972 3). Normalized expression (mean ± SEM) for all 4 stages is presented for each
973 sequence. Solid lines indicate time during anoxia, while dotted lines denote
974 aerobic recovery. (**A,B,C**) Sequences differentially expressed in D2 embryos.
975 (**D,E,F**) Sequences with increased expression during recovery in 4 dpd embryos.
976 (**G,H,I**) MitosRNAs derived from tRNA fragments that increase in abundance
977 during anoxia in 4 dpd embryos. (**J,K,L**) Sequences that are putative anoxic
978 preconditioning responsive in 12 dpd embryos. (**M,N,O**) Expression patterns of
979 sequences for 20 dpd embryos. EA = early anoxia; LA = late anoxia; ER = early
980 recovery; LR = late recovery. See Table 1 for sampling times that correspond to
981 these samples.
982

Figure 6. 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 (gray scale) 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 3. cluster 4-2). The inner pie chart (gray scale) 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 to their representation in the whole catalog.


A
B
RNA yield (ng/embryo)
6000
4000
2000
0
20 dpd
12 dpd
4 dpd
D2
A
B
C
D
D2
4 dpd
12 dpd
20 dpd
Developmental Stage
t=0
EA
LA
ER
LR
Treatment

A


D2
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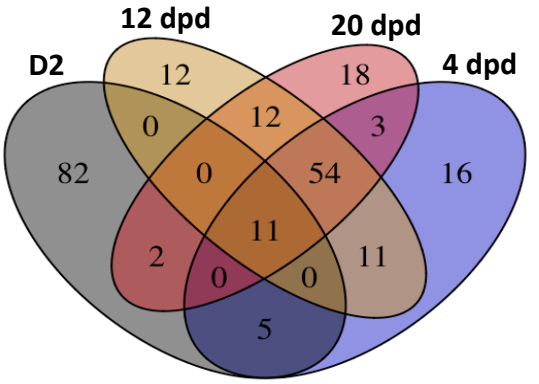
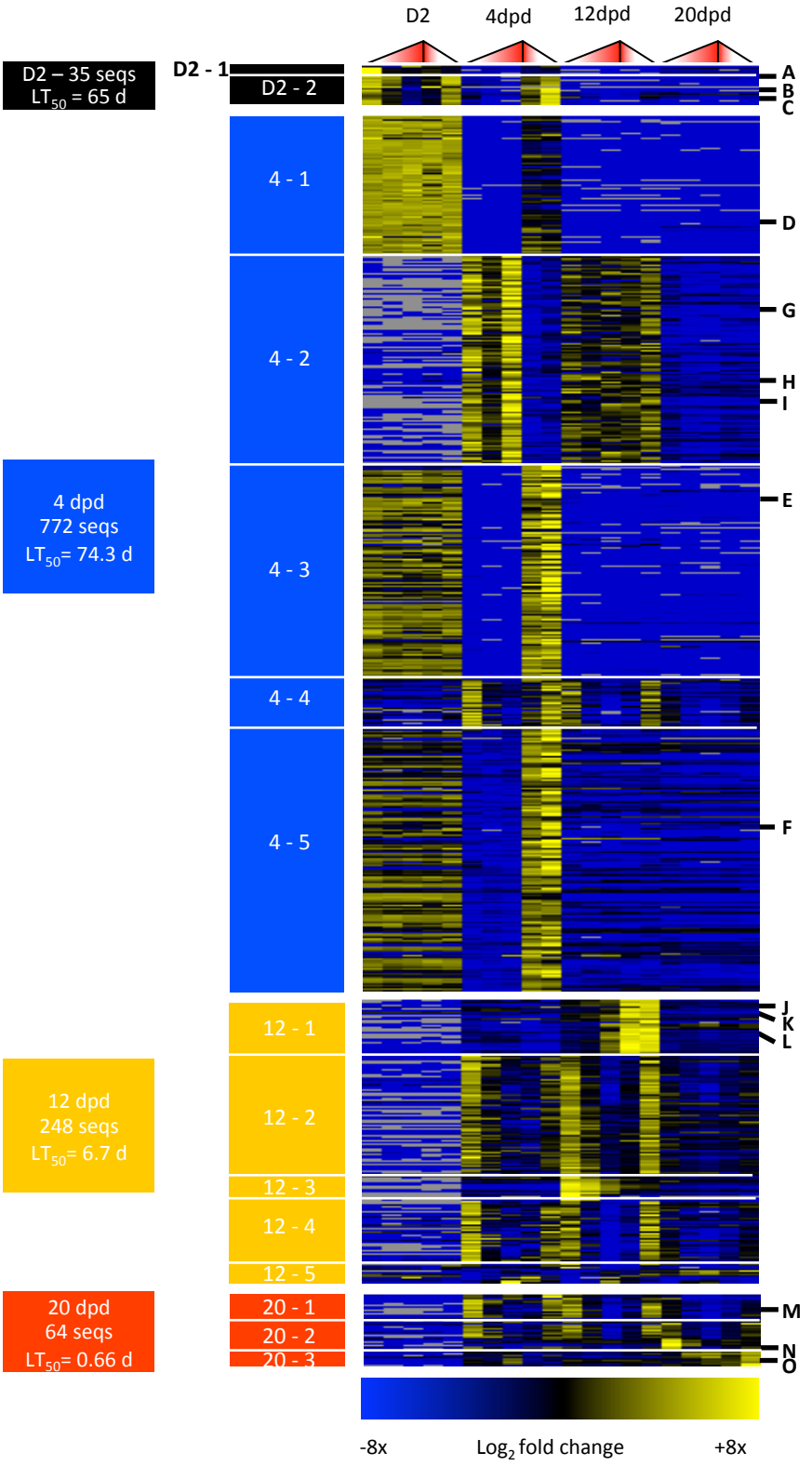
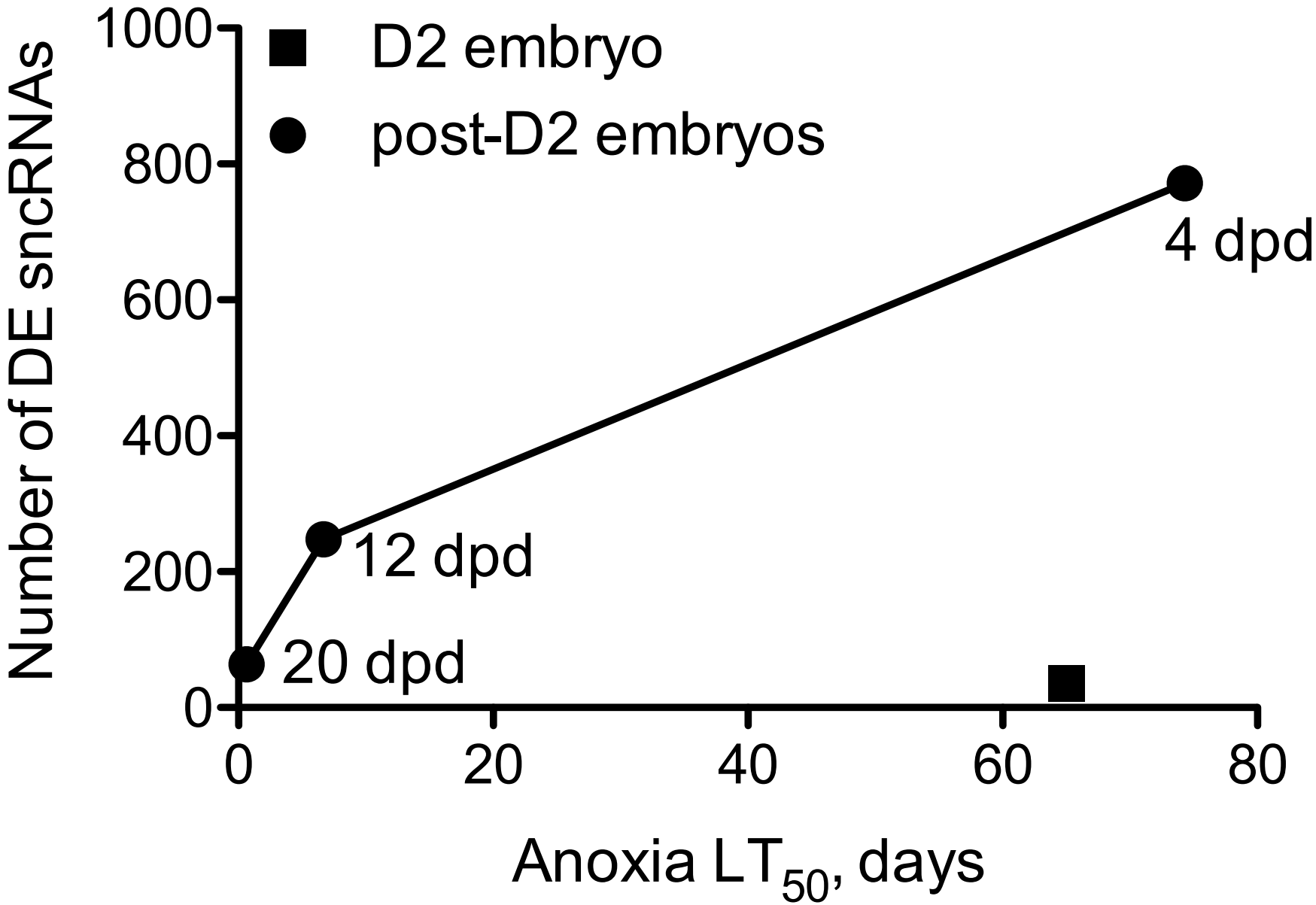
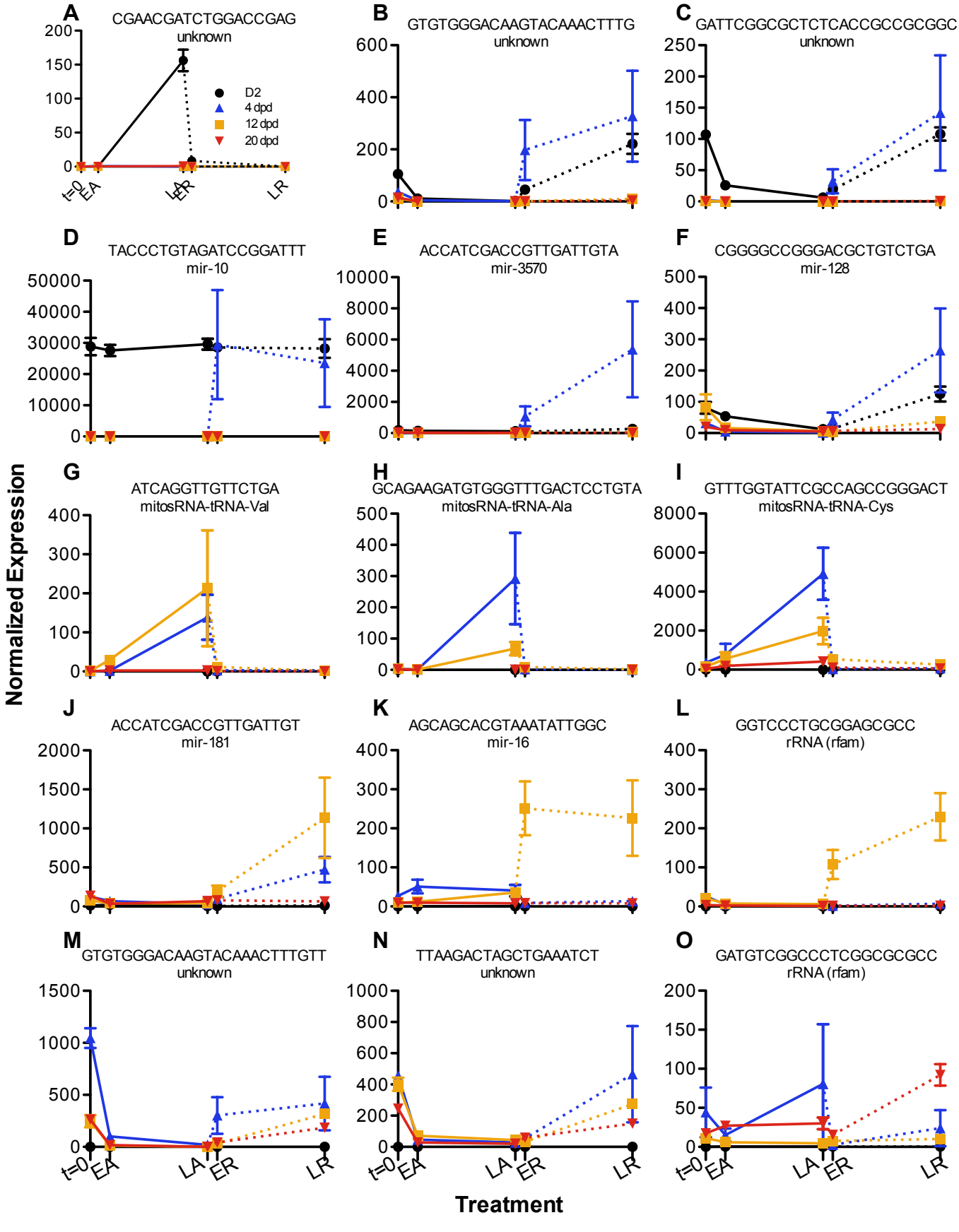
12dpd
20dpd
1
2
3
4
5
-8x
Log2 fold change
+8x

B


D2
12 dpd
20 dpd
4 dpd
82
12
0
18
12
3
0
54
16
11
2
0
0
11
5


D2
4dpd
12dpd
20dpd
D2 – 35 seqs
LT50 = 65 d
D2 - 1
D2 - 2
A
B
C
4 - 1
D
4 - 2
G
H
I
4 dpd
772 seqs
LT50= 74.3 d
4 - 3
E
4 - 4
4 - 5
F
12 - 1
J
K
L
12 dpd
248 seqs
LT50= 6.7 d
12 - 2
12 - 3
12 - 4
12 - 5
20 dpd
64 seqs
LT50= 0.66 d
20 - 1
M
20 - 2
N
20 - 3
O
-8x
Log2 fold change
+8x


1000
800
600
400
200
0
Number of DE sncRNAs
D2 embryo
post-D2 embryos
4 dpd
12 dpd
20 dpd
0
20
40
60
80
Anoxia LT50, days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unknown
B
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unknown
C
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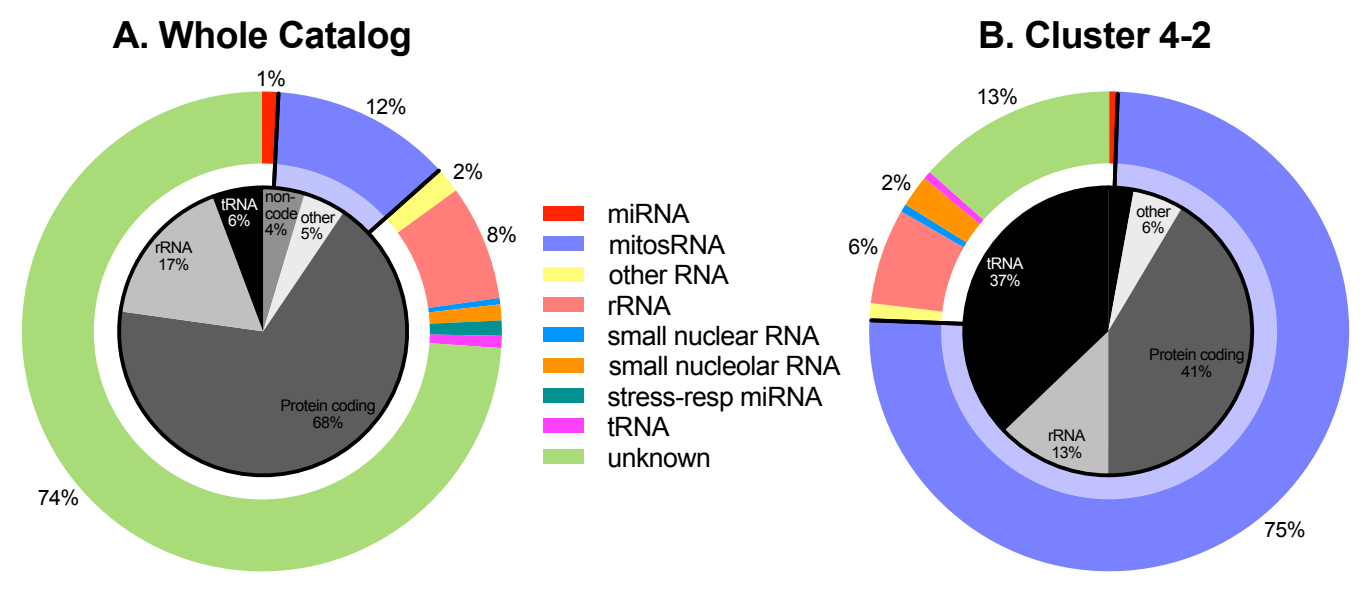
unknown
D
TACCCTGTAGATCCGGATTT
mir-10
E
ACCATCGACCGTTGATTGTA
mir-3570
F
CGGGGCCGGGACGCTGTCTGA
mir-128
G
ATCAGGTTGTTCTGA
mitosRNA-tRNA-Val
H
GCAGAAGATGTGGGTTTGACTCCTGTA
mitosRNA-tRNA-Ala
I
GTTTGGTATTCGCCAGCCGGGACT
mitosRNA-tRNA-Cys
J
ACCATCGACCGTTGATTGT
mir-181
K
AGCAGCACGTAAATATTGGC
mir-16
L
GGTCCCTGCGGAGCGCC
rRNA (rfam)
M
GTGTGGGACAAGTACAAACTTTGTT
unknown
N
TTAAGACTAGCTGAAATCT
unknown
O
GATGTCGGCCCTCGGCGCGCC
rRNA (rfam)
D2
4 dpd
12 dpd
20 dpd
t=0
EA
LA
ER
LR
Normalized Expression
Treatment


A. Whole Catalog
1%
12%
2%
8%
74%
tRNA 6%
non-code 4%
other 5%
rRNA 17%
Protein coding 68%
miRNA
mitosRNA
other RNA
rRNA
small nuclear RNA
small nucleolar RNA
stress-resp miRNA
tRNA
unknown
B. Cluster 4-2
13%
2%
6%
75%
other 6%
tRNA 37%
Protein coding 41%
rRNA 13%