Unique transcriptional signatures of sleep loss across independently evolved cavefish populations

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Abstract

Animals respond to sleep loss with compensatory rebound sleep, and this is thought to be critical for the maintenance of physiological homeostasis. Sleep duration varies dramatically across animal species, but it is not known whether evolutionary differences in sleep duration are associated with differences in sleep homeostasis. The Mexican cavefish, *Astyanax mexicanus*, has emerged as a powerful model for studying the evolution of sleep. While eyed surface populations of *A. mexicanus* sleep approximately eight hours each day, multiple blind cavefish populations have converged on sleep patterns that total as little as two hours each day, providing the opportunity to examine whether the evolution of sleep loss is accompanied by changes in sleep homeostasis. Here, we examine the behavioral and molecular response to sleep deprivation across four independent populations of *A. mexicanus*. Our behavioral analysis indicates that surface fish and all three cavefish populations display robust recovery sleep during the day following nighttime sleep deprivation, suggesting sleep homeostasis remains intact in cavefish. We profiled transcriptome-wide changes associated with sleep deprivation in surface fish and cavefish. While the total number of differentially expressed genes was not greater for the surface population, the surface population exhibited the highest number of uniquely differentially expressed genes than any other population. Strikingly, a majority of the differentially expressed genes are unique to individual cave populations, suggesting unique expression responses are exhibited across independently evolved cavefish populations. Together, these findings suggest sleep homeostasis is intact in cavefish despite a dramatic reduction in overall sleep duration.

Introduction

Sleep is regulated by homeostatic drive and circadian gating (Archer & Oster, 2015; Borbély, Daan, Wirz-Justice, & Deboer, 2016). Homeostatic sleep pressure increases throughout the day for diurnal animals, while circadian drive aligns periods of rest and activity with light-dark cycles (Archer & Oster, 2015; Borbély et al., 2016). Acute or chronic sleep loss induces recovery sleep, suggesting the presence of a sleep homeostat. While the presence of a rebound in response to sleep deprivation has been described in animals ranging from jellyfish to humans (Anafi, Kayser, & Raizen, 2018; Keene & Duboue, 2018; Libourel et al., 2018; Nath et al., 2017), surprisingly little is known about the molecular basis of this homeostat and even less is known about resiliency of different genotypes to insufficient sleep (Diessler et al., 2018). Further, it is unclear whether the genetic differences that underlie the robust differences in sleep duration are linked to sleep homeostasis or resiliency to insufficient sleep (Keene & Duboue, 2018).

Understanding the functional relationship between sleep duration, homeostasis, and resiliency to insufficient sleep would be aided by investigating the relationship between evolved sleep loss and sleep homeostasis. The Mexican tetra, *Astyanax mexicanus*, has emerged as a leading model for investigating the evolution of sleep (Jaggard et al., 2017; Jaggard et al., 2018; Keene, Yoshizawa, & McGaugh, 2015). *Astyanax mexicanus* are a single species consisting of eyed surface fish that inhabit the rivers of northeast Mexico and Texas and at least 30 populations of conspecific blind, cave-dwelling fish (Bradic et al., 2012; Coghill et al., 2014; Espinasa, Rivas-Manzano, & Pérez, 2001; Herman et al., 2018). Several independent origins of the cave phenotype arose within the past ~200k years and effective population sizes of independent caves suggest that selection may be an efficient force in shaping cave-derived traits (Bradic et al., 2012; Coghill et al., 2014; Herman et al., 2018). Sleep is reduced compared to surface fish in five cave populations studied to date, suggesting the evolutionary convergence on sleep loss (Duboué, Keene, & Borowsky, 2011; Jaggard et al., 2018; Yoshizawa et al., 2015). Further, distinct neural mechanisms lead to sleep loss between populations, indicating sleep loss evolved independently in different cavefish populations (Jaggard et al., 2017; Jaggard et al., 2018).

Despite this headway in understanding the evolution of sleep duration, the relationship between changes in evolved sleep duration, homeostasis, and cognition and physiological

resiliency to insufficient sleep is unclear. Previously, it was reported that one cavefish population displayed a rebound following deprivation and the other tested populations approached significance (Duboué et al., 2011). Given the robust evolutionary changes in sleep duration across populations, it is not clear whether rebound sleep in cavefish is dependent on shared or divergent molecular mechanisms from surface fish. Here, we employ a new method of acute sleep deprivation and find surface fish and three cavefish populations display robust sleep rebound following a single night of deprivation.

A recently sequenced genome in *A. mexicanus* allows for genome-wide analysis of context-dependent changes in gene-expression (McGaugh et al., 2014). While transcriptomic approaches have been used to identify developmentally-specified changes in gene expression in *A. mexicanus* (Gross, Furterer, Carlson, & Stahl, 2013; Hinaux et al., 2013; Stahl & Gross), they have not been applied to identify novel regulators of behavior. The power of comparing between independent populations of cavefish with robust differences in sleep behavior, allows for investigation of mechanisms underlying sleep homeostasis as well as physiological and metabolic responses to sleep deprivation.

Here we compare the behavioral and molecular signatures of sleep deprivation in surface fish and three populations of cavefish. We find that recovery sleep is maintained in cavefish populations despite robust evolutionary loss of sleep duration in undisturbed conditions. Transcriptome analysis revealed unique signatures of sleep deprivation suggesting distinct responses to insufficient sleep across all populations.

Methods

Animal Husbandry

Animal husbandry was carried out as previously described (Borowsky, 2008), and all protocols and procedures for this experiment were approved by the IACUC Florida Atlantic University (Protocols A16-04 and A17-32). Fish were housed in the Florida Atlantic University core facilities at 23 ± 1°C constant water temperature throughout rearing for behavior experiments (Borowsky, 2008). Lights were kept on a 14:10 hr light-dark cycle that remained constant throughout the animal's lifetime. Light intensity was kept between 25–40 Lux for both rearing and behavior experiments. All fry were housed in 250 mL pyrex dishes (0-10 dpf, days post fertilization) or 1L tanks (11-30 dpf) and fed *Artemia* twice daily.

100

101 Sleep deprivation

102 We conducted the behavioral and RNA-seq experiments with three different cave
103 populations: Pachón, Tinaja, and Molino and one surface population. Surface and cave fry at 30
104 dpf exhibit differences in sleep duration and architecture (Duboué et al., 2011; Jaggard et al.,
105 2018), thus this is an appropriate stage to assay for variation in sleep rebound. Sleep deprivation
106 was performed by placing fish in 250mL Erlenmeyer flasks on a VWR 2500 multi-vortexer with
107 settings of "4" for speed and "2" for duration. Approximately 20 fry per treatment and
108 population aged 29 dpf were shaken at random intervals < 60 seconds apart since the behavioral
109 definition of sleep is a quiescent period of 60 seconds or more (Duboué, Borowsky, & Keene,
110 2012; Yoshizawa, 2015). This deprivation paradigm lasted throughout the night hours between
111 ZT14-24 on the night before fish were placed into wells for behavioral recordings the following
112 day. Control animals were held under similar conditions as sleep deprived animals but were not
113 shaken.

114

115 Sleep behavior analysis

116 sleep deprivation treatment consisted of shaking beakers of fish randomly, at a consistent
117 speed one time per minute throughout a period of 10 hours during the night (ZT 14-24 8pm to
118 6am). Starting at ZT14, fish in the sleep deprivation treatment were shaken in 200 ml beakers,
119 followed by immediate transfer to 12-well tissue culture plates (BD Biosciences). Control fish
120 were housed under identical conditions but were not shaken. Sleep behavior was recorded in
121 juvenile fish aged 30dpf as previously described (Jaggard et al., 2017; Jaggard et al., 2018).
122 Recording chambers were lit with custom infrared LEDs placed beneath the fish (Jaggard et al.,
123 2019). Videos were recorded using VirtualDub software (v 1.10.4) and were analyzed using
124 Ethovision XT 9.0. All water chemistry was monitored and maintained at standard conditions.
125 Ethovision tracking parameters were set as previously described, and data was processed using
126 custom Perl and Excel scripts (Jaggard et al., 2017; Yoshizawa, 2015). All behavioral recordings
127 were started at ZT0 and recorded for 24 hours. Fish were fed at the start of the behavioral
128 analysis (ZT0). For each treatment and population, 18 fish were analyzed.

129

130 Sample collection for RNA-seq after sleep deprivation

RNA-seq samples were collected from *Astyanax mexicanus* that had been reared in the Keene laboratory at Florida Atlantic University for multiple generations. We conducted the experiment with the same three different cave populations and surface population that were used for the behavioral experiment. Surface parental fish were derived from wild-caught Río Choy stocks originally collected by William Jeffery. Cavefish stocks were Pachón #20, Tinaja 51, and Molino 187.

To minimize non-specific variation, all individuals from each population were collected from the same population-specific clutch (Pachón cave: fertilized from a mating cluster of six parents, Molino cave: from a mating cluster of eight parents, Tinaja cave: from a mating cluster of three parents, Río Choy surface: from a mating cluster of eight parents). Individuals from each population were raised for 27 dpf under standard conditions, (14:10 light-dark cycle), and three days prior to the experiment, fish were transferred into dishes with 12-21 fish per dish in a 14:10 light-dark cycle. Fish were fed ad libitum twice daily in the morning and evening. On days that were non-experiment days, feedings were within a 2-3h window to prevent fish from becoming food-entrained. On experiment days, fish were fed at Zeitgeber time 0 and 12 and were fasted overnight.

As described above, the sleep deprivation treatment consisted of shaking beakers of fish randomly, at a consistent speed one time per minute throughout a period of 10 hours during the night (ZT 14-24, 8pm to 6am). Fish were collected at ZT24 when fish were 30dpf. All individuals were immediately flash frozen in liquid nitrogen upon collection, and stored at -80°C (Figure 1).

For the Molino and Pachón populations, control fish were from a spawning of the same parental fish as sleep deprived fish and were exactly 30dpf when sampled, however they were sampled from a clutch that was spawned approximately a week after the sleep deprived treatment fish. We sampled control fish on the same day of as the sleep deprivation fish, but due to a technical error, we could only sequence one control individual from Molino and Pachón from the same sampling day as the sleep deprived fish. We used this single control sample from Molino and Pachón to determine whether collection days drove a substantial amount of variation. MDS plots revealed that there was little difference between these two sampling times (Figure S1).

RNA extraction, library preparation and sequencing

Protocols for RNA extraction, library preparation, and sequencing were performed as previously described (Passow et al., 2019). To isolate RNA, whole 30dpf fry (~ 30 mg of tissue) were homogenized and then lysed. Total RNA was extracted using the Qiagen RNAeasy Plus Mini Kit (Qiagen) and then quantified (ng/uL) using NanoDrop Spectrophotometer (Thermo Fisher Scientific), Ribogreen assay (Thermo Fisher Scientific), and Bioanalyzer RNA 6000 Nano assay (Agilent). All individuals were extracted within a week by same researcher. The extraction batch was randomized across populations and treatments (Table S1).

All cDNA libraries were constructed at the University of Minnesota Genomics Center on the same day in the same batch, and 400ng of total RNA was used for mRNA isolation and cDNA synthesis. Strand-specific cDNA libraries were then constructed using TruSeq Nano Stranded RNA kit (Illumina), following manufacturer protocol. Before libraries were pooled for sequencing, library quality was assessed using Agilent DNA 1000 assay on a Bioanalyzer. Only two samples exhibited RIN scores < 9 from the extracted total RNA (Table S1).

To minimize sequencing lane effects, barcoded libraries were pooled with treatment and population spread evenly between lanes. Samples were sequenced across multiple lanes of an Illumina HiSeq 2500 to produce 125-bp paired-end reads at University of Minnesota Genomics Center (Table S1). Little variation was explained by extraction batch or lane of sequencing (Passow et al., 2019). All sequence data were deposited in the short read archive (Table S1).

Gene expression quantification

The pipelines used to filter and quantify patterns of differential expression were similar to previous published work (Passow et al., 2019), with minor adjustments. In brief, the raw RNA-seq reads were quality checked using Fastqc (Andrews, 2014) and then trimmed to remove adapters and poor quality reads using the program Trimmomatic v0.33 (Bolger, Lohse, & Usadel, 2014). Trimmed reads were then mapped against the *Astyanax mexicanus* Pachón reference genome (version 1.0.2; GenBank Accession Number: GCA_000372685.1; McGaugh et al., 2014) using the splice-aware mapper STAR (Dobin et al., 2013). Stringtie v1.3.3d (Pertea et al., 2016; Pertea et al., 2015) was used to quantify the number of reads mapped to each gene in the reference *A. mexicanus* genome annotation. Finally, to generate the counts matrix, we used the python script provided with Stringtie (prepDE.py; Pertea et al., 2016). Annotations were

192 extracted from the *Astyanax mexicanus* annotation file from Ensembl
193 (Astyanax_mexicanus.AstMex102.91.gtf).
194
195 Variation in gene expression
196 For quality control, each gene was required to have greater than two counts per million
197 (cpm) and to have counts data for three or more individuals across the sleep deprivation and
198 control samples for all populations. This resulted in 17,187 genes that were analyzed. We
199 identified genes that showed the largest difference in observed gene expression between sleep
200 deprived and standard conditions using the Bioconductor package, EdgeR (Robinson, McCarthy,
201 & Smyth, 2010). We used the calcNormFactors command on our DGEList, and generated
202 multidimensional scaling plots of the top genes using the gene selection method "common."
203 Given tagwise dispersion and a design matrix, we used glmFIT to fit a negative binomial GLM
204 for each tag and DGEGLM object to perform likelihood ratio test based on the contrast matrix of
205 comparisons. To perform a principal component analysis, we also utilized the log-cpm values of
206 the counts data with the function prcomp(). P-values for differential expression were adjusted
207 based on the Benjamini-Hochberg algorithm, using a default false discovery rate of 0.05 (Love *et*
208 *al.* 2014). Genes were labeled as differentially expressed if the Benjamini-Hochberg adjusted P-
209 value was less than 0.05 (Tables S2-S5). Log2(Sleep deprived/standard) values were calculated
210 with DESeq2 and exported for further analysis.
211
212 Annotation of differentially expressed genes
213 We conducted annotation analyses using differentially expressed genes at the 0.05 false
214 discovery rate using PANTHER analysis (Mi *et al.* 2016)
215 (http://pantherdb.org/tools/compareToRefList.jsp). PANTHER analysis was run using only 1:1
216 orthologs between zebrafish and *Asytanax* with database current as of 2018-12-03. Within the
217 PANTHER suite, we used PANTHER v14.0 overrepresentation tests (i.e., Fisher's exact tests
218 with FDR multiple test correction) with the Reactome v65, PANTHER proteins, GoSLIM, GO,
219 and PANTHER Pathways. The target list was the zebrafish genes that were 1:1 orthologs to
220 differentially expressed *Astyanax* genes, and the background list was the 1:1 zebrafish orthologs
221 of *Astyanax* genes analyzed for differential expression.
222

Selection analyses

We cross-referenced differentially expressed genes to genes likely under selection using the same analysis metrics detailed in (Herman et al., 2018; Yoshizawa et al., 2018). For population genomic measures, we included a core set of whol genome resequenced samples which contained: Pachón cave, N = 10 (9 newly resequenced + the reference reads mapped back to the reference genome); Tinaja cave, N =10; Molino cave, N = 9; Rascon surface, N = 6; and Río Choy surface, N = 9. We required six or more individuals have data for a particular site. For all population genomic measures, we excluded masked repetitive elements, indels (if present in any of the core set of samples), and 10bp surrounding the bases affected by each indel by using the masking_coordinates.gz file available for the *Astyanax mexicanus* genome v1.0.2 though NCBI genomes FTP.

Our procedures for outlier analyses have been described (Yoshizawa et al., 2018), but we briefly recap here. We used hapFLK v1.3 https://forge-dga.jouy.inra.fr/projects/hapflk (Fariello et al., 2013) for genome-wide estimation of the hapFLK statistic of across all 44 *Astyanax mexicanus* samples and two *Astyanax aeneus* samples. HapFLK accounts for hierarchical population structure by building local ancestry trees and detects changes in haplotype frequencies which exceed what is expected for neutral evolution (Fariello et al., 2013). HapFLK outperformed many other statistics (Schlamp et al., 2016) and may be robust to bottlenecks and migration. We augment this measure with metrics of F_{ST}, π, and d_{XY}. We also required that π that was not excessively low in the two surface populations, Rascon and/or Río Choy, (rank of greater than 500 for dense ranking of genes or lowest ~2.5% of diversity values in the genome) to protect, in part, against calling outliers from inflated relative measures of divergence that were driven by low diversity due to low recombination or sweeps in the surface population. As we are interested in identifying genes under strong selective sweeps in the cave populations, we did not want to exclude genes with low diversity in cave populations. F_{ST} is more sensitive to changes in allele frequency than absolute measures such as d_{XY} (Cruickshank & Hahn, 2014).

Results

To identify expression signatures of sleep deprivation, we performed RNA-seq on surface fish and three populations of cavefish. At 29dpf fish were sleep deprived for 10hrs by mechanical

shaking shown to elicit sleep rebound. Fry were sampled for behavioral sleep rebound and RNA-seq at 30dpf.

Sleep homeostasis behavioral assays

To quantify sleep homeostasis across *A. mexicanus* populations, we sleep deprived fish throughout the night period (ZT14-24) and compared sleep the following day to undisturbed controls (Figure 2A). Surface fish, as well as Pachón, Molino, and Tinaja cavefish displayed a significant increase in daytime sleep following deprivation suggesting sleep homeostasis is intact in all three cavefish populations (Figure 2B, D-G). When sleep measurements were extended into the night following deprivation (ZT14-24), total sleep duration did not differ between control and sleep deprived fish across all four populations tested (Figure 2C, D-G), suggesting the recovery sleep during the day was sufficient to return sleep drive to levels in undisturbed fish. Taken together, these findings reveal sleep homeostasis is intact across three populations of cavefish, despite exhibiting robustly diminished basal sleep levels.

Mapping statistics

On average we sequenced 16.72 million reads per sample (median = 16.77 million, min = 13.36, max = 19.88) from whole, individuals at 30 dpf (Table 1, Table S1, Supplemental materials). Pachón cavefish exhibited slightly higher percentage of reads mapped compared to all other populations, as would be expected given reference sequence bias. Total yield of reads were not significantly different between treatments (Wilcoxon test W = 252.5, p-value = 0.4705). On average, 88.96% reads mapped per sample to the *Astyanax mexicanus* genome (median = 88.9%, range 85.06% - 91.59%). Filtering of the gene counts matrix to include only transcripts with greater than two cpm and three or more individuals with counts data resulted in 17,187 genes used for differential expression analysis and clustering analysis, except where otherwise noted. Libraries were normalized by size using calcNormFactors (Figure S2), and samples did not exhibit clustering due to lane of sequencing or extraction batch (Figure S3, S4).

General patterns in expression response to sleep deprivation

After correcting for multiple testing, the number of significantly differentially expressed genes between sleep deprived and control groups is < 2.1% of the 17,187 genes used for differential

expression analyses in all populations (Table 1). Multidimensional scaling analysis showed that the major axis of differentiation among the top 500 genes was ecotype (Figure 3) and explained 33% of the variation, suggesting that expression profiles of cave populations are more similar to each other than to surface fish. The second principal component accounted for 12% of the variation in the data and separated the caves into two clusters containing 1) Pachón-Tinaja and 2) Molino, which represent two separate lineages of cavefish (Herman et al., 2018). Thus, ecotype and population (and not sleep deprivation treatment) drove the majority of variation in the complete dataset. Since population is such a strong driver of expression, to isolate the effect of sleep deprivation on expression profiles, we separated the populations into individual MDS plots (Figure S5, S6, S7, S8).

Across population-specific MDS plots, sleep deprivation treatment accounted for 12-16% of the variation in the dataset. However, individual populations exhibited different signatures. Most notably, Pachón cave population exhibits little differentiation between sleep deprived and control samples (Figure S5) compared to the other populations (Figure S6, S7, S8).

Divergent responses across cave and surface populations in response to sleep deprivation

Despite surface fish exhibiting greater than 3-fold longer total sleep than cavefish at this stage of development (Duboué et al., 2011and Figure 1), the 10-hour sleep deprivation treatment did not result in a significant increase in the total number of differentially expressed genes in surface fish compared to any of the cave populations. Surface fish exhibited ~1.6 and ~2.2 fold the number of upregulated genes in response to sleep deprivation than Molino and Pachón cavefish, respectively (Table 1). Surface fish also experienced ~1.5 fold the number of downregulated genes than Pachón cavefish (Table 1). However, Tinaja cavefish exhibited a greater total number of upregulated genes than the three other populations (Table 1). Thus, we did not observe a direct pattern that evolved sleep loss in cave populations results in fewer differentially expressed genes in response to sleep deprivation, fortifying the behavioral findings that sleep homeostasis is largely intact in cavefish population.

While surface fish did not exhibit more exacerbated transcriptional responses to sleep deprivation than cavefish, the surface population exhibited the highest number of uniquely differentially expressed genes than any other population (83.5-86.7%, Table 1), suggesting surface-specific responses to sleep deprivation. More cave-specific overlap in differentially

315 expressed genes, though, could be because the analyses included more cave populations than
316 surface populations.
317
318 Population-specific changes in response to sleep deprivation
319 Across all cave and surface populations, more differentially expressed genes were found to be
320 shared than estimated by chance, suggesting a degree of conserved responses to sleep
321 deprivation. Between cave populations, the number of concordant upregulated and
322 downregulated genes was significantly greater than random for all comparisons ($p << 0.0001$;
323 Figure 3). Between cave and surface comparisons, there was significant overlap, but to a lesser
324 degree than in comparisons considering only cave populations ($p \leq 0.007$, for all cave-surface
325 comparisons). As would be expected, the number of genes showing discordant patterns
326 (upregulated in one population and downregulated in another) was not significantly different
327 from random for all pairwise-population comparisons ($p > 0.145$ in all cases). Therefore, across
328 populations more genes were overlapping in concordant transcriptional response than expected
329 by chance alone.
330 While the overlap was significantly more than expected by chance, few differentially
331 expressed genes were shared between populations. For example, between 5.0-7.8% of
332 differentially expressed genes overlapped in the same direction among pairwise comparisons of
333 caves, and 1.4-2.4% of differentially expressed genes overlapped across all three cave
334 populations. While we found significant overlap in expression responses between populations,
335 67-82% of differentially expressed genes are unique to specific cave populations. This lends
336 support to the hypothesis that response to sleep deprivation and sleep homeostatic processes may
337 exhibit strong intraspecies differences among caves.
338
339 Functional enrichment analysis
340 Very few functional categories were significantly enriched after correction for false discovery
341 rate across all populations (FDR; Tables S6-S13). For cave populations, gene ontology analyses
342 indicated that upregulated genes were significantly enriched for oligopeptide transmembrane
343 transport activity (Pachón) and intermediate filaments cytoskeleton organization associated with
344 wound healing (Tinaja). One of the consistently upregulated genes in past mammalian studies of
345 sleep homeostasis is *activity-regulated cytoskeletal associated protein* (*arc*) (Diessler et al.,

2018). While this gene is not annotated in any fish genome on Ensembl, it is notable that cytoskeleton-related processes are enriched in genes upregulated in response to sleep deprivation in Tinaja cavefish. Downregulated genes for cave populations were associated with winged helix/forkhead transcription factors in Molino ($p = 0.054$, after FDR correction) and nitrogen compound transport in Tinaja. In the surface population, upregulated genes are significantly enriched for transmembrane transport functions of a variety of molecules, but downregulated genes exhibit no significant functional enrichment. The different GO categories for each population support the notion that sleep deprivation evokes variable intraspecific physiological responses across different *A. mexicanus* populations.

Selection analyses

Several differentially expressed genes also exhibit signatures of positive selection (Table S14). Notably, *aif1l* (*allograft inflammatory factor 1 like*) is downregulated in Molino and Pachón cavefish in response to sleep deprivation and exhibited population genetic parameters suggestive of a recent selective sweep in both populations (e.g., significant with HapFLK analyses, in the top 5% of F_{ST} values across the genome between surface and Molino populations, in the top 10% of F_{ST} values across the genome between surface and Pachón populations, and one of the least diverse genes across the genome in both cave populations). Knockout mice of *aif1l* exhibited deformities in endocrine/exocrine, hematopoietic, immune, integument, and liver/biliary systems (Dickinson et al., 2016), suggesting a pleiotropic link between extended wakefulness and liver and immune function in cavefish. Next, *slc17a8* is downregulated in Tinaja cavefish in response to sleep deprivation and is involved in equilibrioception, neuromast hair cells, neuron-neuron synaptic transmission, posterior lateral line neuromast hair cell development, startle response, and vestibular reflex (Obholzer et al., 2008). Population genetic parameters of *slc17a8* suggest a recent selective sweep in Tinaja (significant with HapFLK, in the top 5% F_{ST} outliers between Tinaja cave and Rascon surface, while being among the lowest diversity genes in Tinaja). The down regulation of lateral line components in response to sleep deprivation is especially interesting since sleep duration in at least one cavefish population is restored with the ablation of the lateral line (Jaggard et al., 2017). Our results suggest that dampening signals from the lateral line may be a response to sleep pressure. Interestingly, Pachón cavefish exhibited significant upregulation of the retinal photoreceptor-associated gene *phosphodiesterase 6G* (*pde6gb*) which

is highly enriched in the pineal gland relative to the brain of larval and adult zebrafish (Toyama et al., 2009), suggesting *pde6gb* may play a role in circadian physiology. This gene exhibited metrics of a recent selective sweep in Pachón (e.g. significant with HapFLK analysis, in the largest 20% Dxy and largest 5% Fst for Pachón-surface comparisons and exhibits very low diversity in Pachón). The three cases highlighted here suggest that selection in cave populations may have shaped liver, lateral line, and photoreceptor functioning, and these traits pleiotropically impacted sleep phenotypes or vice versa.

Discussion

While sleep duration is widely studied across animal species, sleep homeostasis and baseline sleep phenotypes are likely functionally distinct (Anafi et al., 2018; Joiner, 2016; Keene & Duboue, 2018). A central question is whether the truncated sleep duration that has evolved in cavefish (Duboué et al., 2011; Jaggard et al., 2018) also impacted sleep homeostasis or whether these processes are independently regulated. Here we implemented an acute sleep deprivation treatment to examine the behavioral and molecular effects in surface fish and three independently-evolved cavefish populations. Our behavioral analysis confirms previous findings that sleep homeostasis is intact across cavefish populations (Duboué et al., 2011), supporting the notion that baseline sleep phenotypes are regulated by separate processes from sleep homeostasis (Allada, Cirelli, & Sehgal, 2017). Sleep significantly deprivation impacted expression of only a few hundred genes across populations, and different cavefish populations and surface fish exhibit discordant gene expression responses to the same sleep deprivation treatment. Thus, our results demonstrate intraspecific variation in molecular mechanisms of sleep pressure in response to sleep deprivation.

Sleep deprivation is an effective way to induce sleep rebound and results in a host of pathophysiological and cognitive deficits (Cappuccio, D'elia, Strazzullo, & Miller, 2010; Cedernaes et al., 2018; Leproult, Holmbäck, & Van Cauter, 2014). Our analysis comparing gene expression in control and sleep deprived fish revealed differential gene expression in only a few hundred genes in response to sleep deprivation across all populations. However, we observed three genes that were differentially expressed across all cave and surface populations, indicating they are part of a generalized response to sleep deprivation: *heat shock protein alpha-crystallin-related, b11* (*hspb11*) and *neutral cholesterol ester hydrolase 1a* (*nceh1a*) were upregulated and

transcription factor *E74-like factor 3* (*elf3*) was downregulated in response to sleep deprivation. Heat shock proteins and other molecular chaperones are often upregulated after sleep deprivation as an indicator of cellular stress, suggesting this is an evolutionarily conserved response to sleep deprivation (Allada et al., 2017; Cedernaes et al., 2018; Mackiewicz et al., 2009; Uyhelji et al., 2018). N*eutral cholesterol ester hydrolase 1a* (*nceh1a*) promotes adipogenesis (Homan, Kim, Cardia, & Saghatelian, 2011), and is strongly implicated in atherosclerotic lesions (Igarashi et al., 2010; Okazaki et al., 2008), indicating that *nceh1a* may be a candidate for mediating the well-documented relationships between short sleep duration and advancement of atherosclerosis and weight gain (Cedernaes et al., 2018; Davies et al., 2014; Levy et al., 2012; Nakazaki et al., 2012). *E74-like factor 3* (*elf3*) is the only gene significantly downregulated in response to sleep deprivation in all populations. Interestingly, this gene is involved in inflammatory response (Conde et al., 2016), epithelial cell differentiation (Kwon et al., 2009), and is a cancer gatekeeper (Gajulapalli et al., 2016; Shatnawi et al., 2014; Yachida et al., 2016; Yeung et al., 2017), suggesting a molecular link between sleep disruption and increased cancer risk (Blask, 2009; Haus & Smolensky, 2013). Together, these genes may be conserved factors that are regulated in accord with sleep drive and deprivation across *A. mexicanus* populations.

All genes that were not differentially expressed in the surface population, but were significantly upregulated across all cave populations were linked to sleep or circadian function. *Carnitine palmitoyltransferase 1B* (*cpt1b*) variants are associated with narcolepsy in humans and upregulated *cpt1b* may increase hypocretin activity (Han et al., 2012; Miyagawa et al., 2008), consistent with previous work demonstrating that increased hypocretin is highly associated with short sleep duration in cavefish (Jaggard et al., 2017; Jaggard et al., 2018). *aarF domain containing kinase 5* (*adck5*), was upregulated across all cave populations in response to sleep deprivation and human variants are associated with insomnia symptoms (Lane et al., 2017). *Telethonin* (*tcap*), a component of cardiac sarcomeres, is regulated by circadian components CLOCK and BMAL1 (Podobed, Alibhai, Chow, & Martino, 2014), supporting the close relationship between sleep homeostasis and circadian cycles suggested by other work (Allada et al., 2017; Borbély et al., 2016). In contrast, none of the genes that were significantly downregulated in response to sleep deprivation across all caves have direct links to sleep or circadian function (e.g. *gamma-glutamyl hydrolase-like, ETS homologous factor*, or *cathepsin K*), though *transmembrane and coiled-coil domain family 1* (*TMCC1*) may potentially be

439 involved in endoplasmic reticulum stress or downregulation of protein synthesis, which are
440 common responses to sleep deprivation (Mackiewicz et al., 2009) (Zhang et al., 2014).
441 After sleep deprivation, five genes were upregulated in surface fish while being
442 downregulated in at least one cave population. In contrast, no genes were downregulated in
443 surface fish while being upregulated in any cavefish population, lending support for the idea that
444 cavefish may be more resilient to extended wakefulness than surface fish. All genes
445 downregulated in Molino cavefish and upregulated in surface fish play critical roles in glucose
446 homeostasis. In mammals, increased expression of *sodium-coupled neutral amino acid*
447 *transporter 3* (*slc38a5*) triggers pancreatic alpha cell proliferation (Kim et al., 2017) which
448 secrete glucagon to elevate the glucose levels in the blood. In response to sleep deprivation,
449 surface fish upregulate *slc38a5a*, which is typically upregulated when glucose levels drop and
450 circulating amino acid levels increase. The ultimate effect of upregulating *slc38a5a* in surface
451 fish is an expected increase in glucagon and subsequently, elevated circulating glucose levels. In
452 contrast to surface fish, Molino cavefish downregulate *slc38a5a* in response to sleep deprivation
453 which would ultimately lead to a decreased in glucagon and subsequently, decreased circulating
454 glucose levels. Next, *proteinase-activated receptor 2-like* (*par2b* aka *f2rl1.2*) is involved in a
455 variety phenotypes, but recent evidence suggests that downregulation of *par2b*, as seen in
456 Molino cavefish, would result in reduction of generation of glucose from non-carbohydrate
457 sources (e.g. gluconeogenesis), ultimately leading to a reduction in circulating glucose levels
458 (Wang et al., 2015). Lastly, knockout *CCAAT/enhancer-binding protein beta-like* (*cebpb*) mice
459 exhibit reduced hepatic glucose production through glycogenolysis (Liu et al., 1999), suggesting
460 that reduced expression in Molino cavefish may result in the reduction of the conversion of
461 glucagon to glucose. Similar to surface fish, *cebpb* is upregulated in response to sleep
462 deprivation in rats (Elliott et al., 2014) (Cirelli, Faraguna, & Tononi, 2006) suggesting increased
463 glucose production. These expression differences are notable as cavefish and surface fish
464 experience different physiological responses to starvation (Jaggard et al., 2017). Surface fish
465 implement sleep deprivation upon starvation, presumably to increase time for food searching,
466 while Molino and Pachón cavefish increase sleep upon starvation, presumably to conserve
467 energy (Jaggard et al., 2017). Future work should investigate the interplay between sleep
468 plasticity and starvation, and our expression data suggest that sleep deprivation upon starvation

469 in surface fish will lead to increased circulating glucose, while sleep deprivation in response to
470 starvation in cavefish would potentially quickly deplete energy stores.
471 Sleep restriction in humans is linked to increased food consumption, weight gain and
472 obesity, insulin resistance, and type II diabetes (Cedernaes et al., 2018; Rao et al., 2015; Zhu et
473 al., 2019). Several genes related to glucose homeostasis were upregulated in cave-specific
474 responses. Genes upregulated in Molino cavefish in response to sleep deprivation included:
475 *pyruvate dehydrogenase kinase 4* (*pdk4*), which is increased in mouse models of insulin
476 resistance and type II diabetes (Cedernaes et al., 2018); *carnitine palmitoyltransferase 1b*
477 (*cpt1b*), which is also involved in insulin resistance; and *leptin receptor*, which is involved in a
478 variety of metabolic phenotypes including obesity. Likewise, in Tinaja cavefish genes
479 upregulated in response to sleep deprivation included *insulin receptor substrate 1* (*irs1*) and
480 s*olute carrier family 2 member 4* (*slc2a4*) which are both strongly associated with glucose
481 homeostasis. Cavefish populations exhibit intraspecific variation for metabolic phenotypes
482 across independently evolved cave populations (Aspiras et al., 2015; Riddle et al., 2018), and the
483 diverse expression changes presented here suggest intraspecific variation in maintaining glucose
484 homeostasis in response to sleep deprivation.
485 Despite known links between sleep homeostasis and circadian regulation on a molecular
486 and genetic level, many circadian and sleep-associated genes are not differentially expressed
487 between fish in the sleep deprivation and control treatments. Little is known about genetic
488 regulation of sleep homeostasis in *A. mexicanus*, but previous work has documented a number of
489 factors that regulate circadian cycling of certain transcripts (Beale et al., 2013) and sleep
490 regulation (Jaggard et al., 2018). For example, Pachón cavefish evolved enhanced levels of the
491 wake-promoting *hypocretin neuropeptide precursor HCRT*, conferring sleep loss in this
492 population (Jaggard et al., 2018). At least in part, this phenotype is developmentally regulated as
493 elevated homeobox transcription factor LHX9 specifies a greater number of HCRT-neurons in
494 cavefish (Alie et al., 2018). While *HCRT* was not expressed at sufficient levels to be analyzed by
495 our differential expression analysis, *lhx9* was not differentially expressed after sleep deprivation
496 across any of the populations examined, supporting the notion that different molecular processes
497 regulate sleep duration under standard conditions and sleep homeostasis. In addition, a number
498 of genes previously identified as regulating sleep in zebrafish and mammals are also not
499 differentially expressed for any tetra population in response to sleep deprivation, despite being

conserved signaling molecules in sleep regulation across the animal phylogeny. These include *aanat2*, an important enzyme for the production of melatonin, gaba receptors (*gabarapa*, *gabarapl2*), brain derived neurotrophic factor (*bdnf*), adenosine receptors (*adora1a*, *adora1b, adora2a*, *adora2b*), adenosine deaminase (*ada*, *adar*), adenosine kinase (*adka*, *adkb*) (Holst & Landolt, 2015), NMDA receptors (e.g., *grin* paralogs, *nsmfa*) (Liu, Liu, Tabuchi, & Wu, 2016), *flotillin* (*flot1*) (Mackiewicz et al., 2007) and dopamine receptors and transporters (*drd1b*, *drd4a*, *drd4b*, *slc6a3*). Likewise, key clock genes (*clockb*, *per1a*, *per1b, per2*, *per3*, *arntl1a*, *arntl1b*, *cry1aa*, *cry1ab, cry1ba*, *cry1bb, cry4*, *roraa*, *rorab*, *rorc*), which are often impacted in sleep deprivation studies (Allada et al., 2017; Archer et al., 2014; Archer & Oster, 2015; Borbély et al., 2016; Franken, 2013; Möller-Levet et al., 2013; Uyhelji et al., 2018), are all not differentially expressed for any tetra population. Together, this suggests that genes regulating sleep duration and circadian function under standard conditions are largely unaffected by a single night of sleep deprivation in tetras.

Several considerations must be taken into account in evaluating our study. First, our study examined the effect of a single night of sleep deprivation in fish housed on a standard 14:10 light cycle. While the results suggest relatively limited changes in the number of differentially expressed genes, it is consistent with studies examining the effects of acute sleep deprivation in other animals. For example, a similar number of genes had altered expression levels in humans after various levels of sleep deprivation (Aho et al., 2013; Cedernaes et al., 2018; Pellegrino et al., 2012). We predict that longer-term sleep deprivation for days or chronic insufficient sleep over a number of days may result in more robust changes in gene expression, however, these protocols would also be likely to induced generalized stress (Pellegrino et al., 2012).

Second, our study employed whole-body sampling for RNA-seq from mRNA transcripts. Tissue specific differences are documented to result from sleep deprivation (Cedernaes et al., 2018) and may obscure signal from specific genes (Diessler et al., 2018). For example, *per2* expression increases in sleep deprived mice and remains elevated for varied amounts of time depending on the tissue (Curie, Maret, Emmenegger, & Franken, 2015). At 30dpf, brain dissection is technically challenging and would likely require pooling across samples. Further, precise dissection of tissue takes time and would result in the samples collected last being sleep deprived for longer than the samples collected first.

To our knowledge, these findings are the first genome-wide analysis of sleep deprivation induced changes in fish. In zebrafish, sleep deprivation robustly impacts cellular processes and behavior (Aho et al., 2017; Elbaz et al., 2017; Pinheiro-da-Silva, Silva, Nogueira, & Luchiari, 2017; Zada et al., 2019), but the effects on large-scale changes in gene expression have not been investigated. Both zebrafish and *A. mexicanus* provide robust models for the identification of genetic and pharmacological regulators of sleep (Duboué et al., 2012; Jaggard et al., 2018; Prober, 2018; Rihel, Prober, & Schier, 2010), suggesting these models can be used to investigate the genetic architecture associated with sleep loss. Our findings, that sleep deprivation induces different molecular signatures in each of the four *A. mexicanus* populations tested, raises the possibility that the response to sleep pressure is highly heterogenous across individuals of the same species. The application of recently developed gene-editing approaches in *A. mexicanus* (Ma et al., 2018; Stahl et al., 2019) combined with the behavioral assay described here may allow for functional validation of these genes and identification of novel regulators of sleep homeostasis.

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Figure 1. Sampling schematic. We sampled three cave populations: Tinaja, Pachón, and Molino and one surface population at 30 days post fertilization. Fish were sleep deprived for 10hrs during their normal dark period through intermittent shaking.

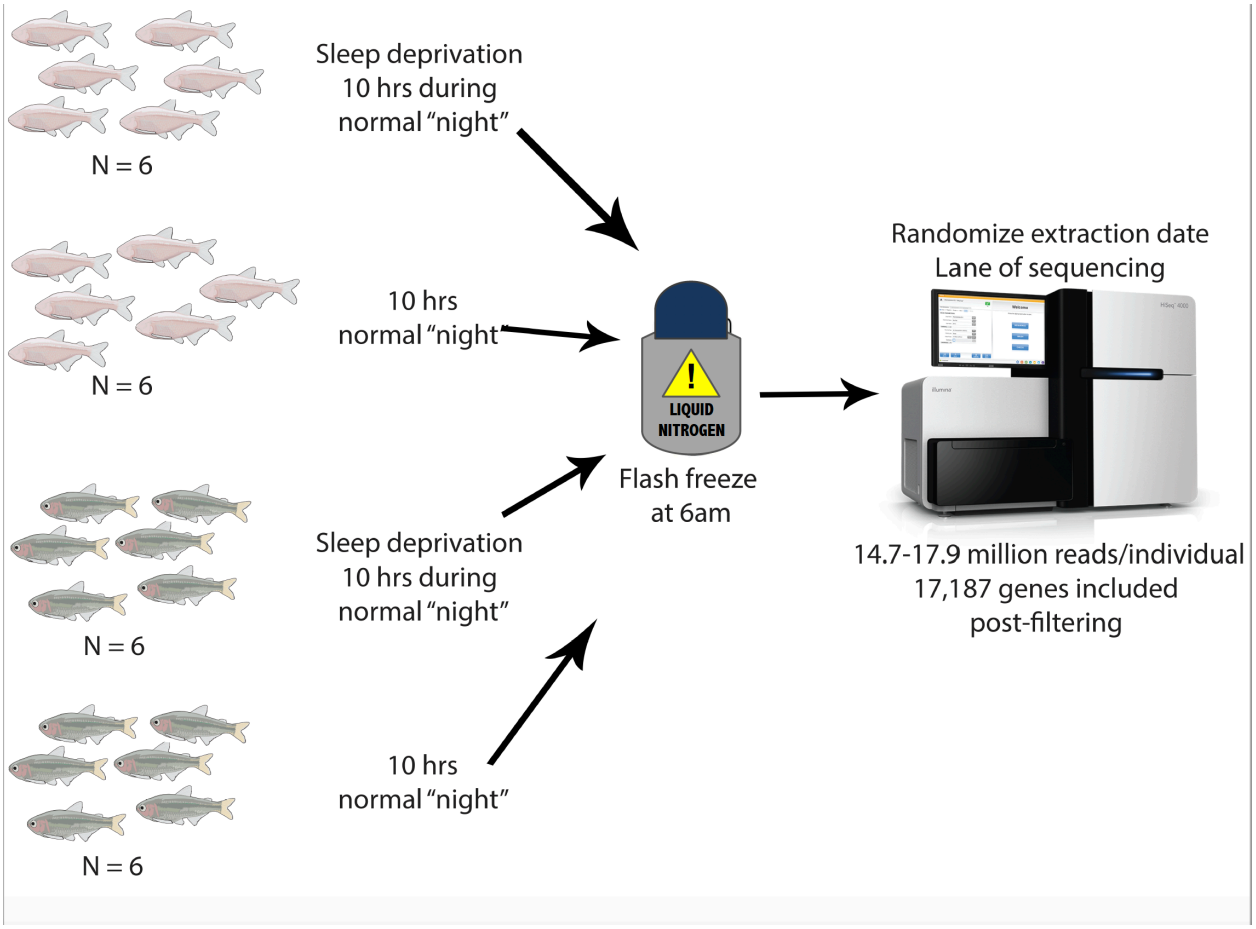

Figure 2. A) Following 14hrs of acclimation, fish were sleep deprived for 10 hours (red line) during the night and behavior was measured over the following 24 hrs. **B)** All four groups of sleep deprived fish (grey) slept significantly more than undisturbed controls (white) during the 14hrs of daytime sleep following deprivation due to longer sleep bouts. **C)** No differences in sleep duration were detected between group during the 10 hrs of night following deprivation. Sleep profiles for **D)** surface, **E)** Pachón, **F)** Tinaja, and **G)** Molino over the day following deprivation. Color lines denote sleep derived fish, black lines represent undisturbed controls. Grey represents standard error of the mean.

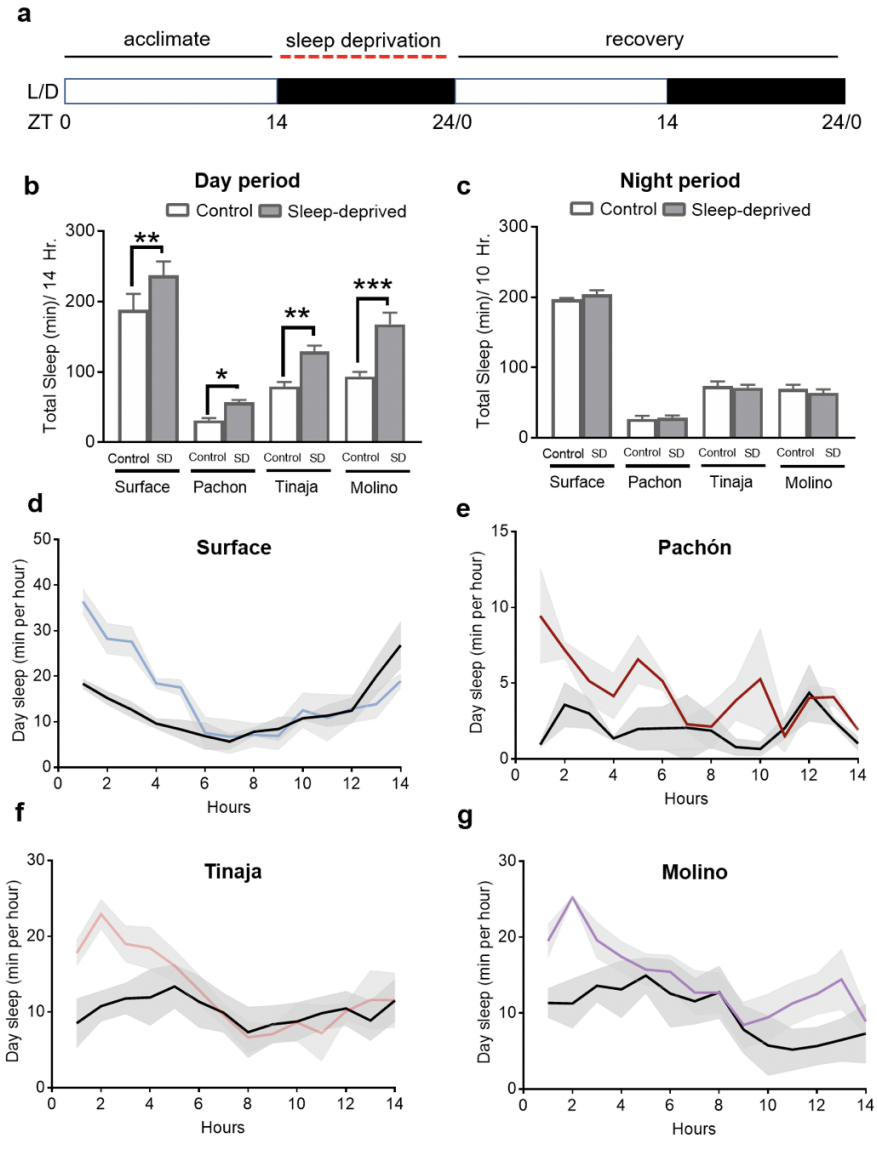

Figure 3. A) MDS plot of 500 Top genes between control and sleep deprivation treatments the four populations normalized for library size. PC1 clearly demarcates population of origin. **B)** Number of genes differentially expressed between control and sleep deprived treatments. Above zero is upregulated in sleep deprived samples and below zero is downregulated in sleep deprived samples. Light grey = shared with at least one other population, Dark grey = not shared among other populations. Overall few genes are differentially expressed in response to sleep deprivation and differentially expressed genes are not shared among populations. **C)** Venn-diagram of overlap in differentially upregulated genes in the sleep deprivation treatment between all four populations. **D)** Venn-diagram of overlap in differentially downregulated genes in the sleep deprivation treatment across all four populations.

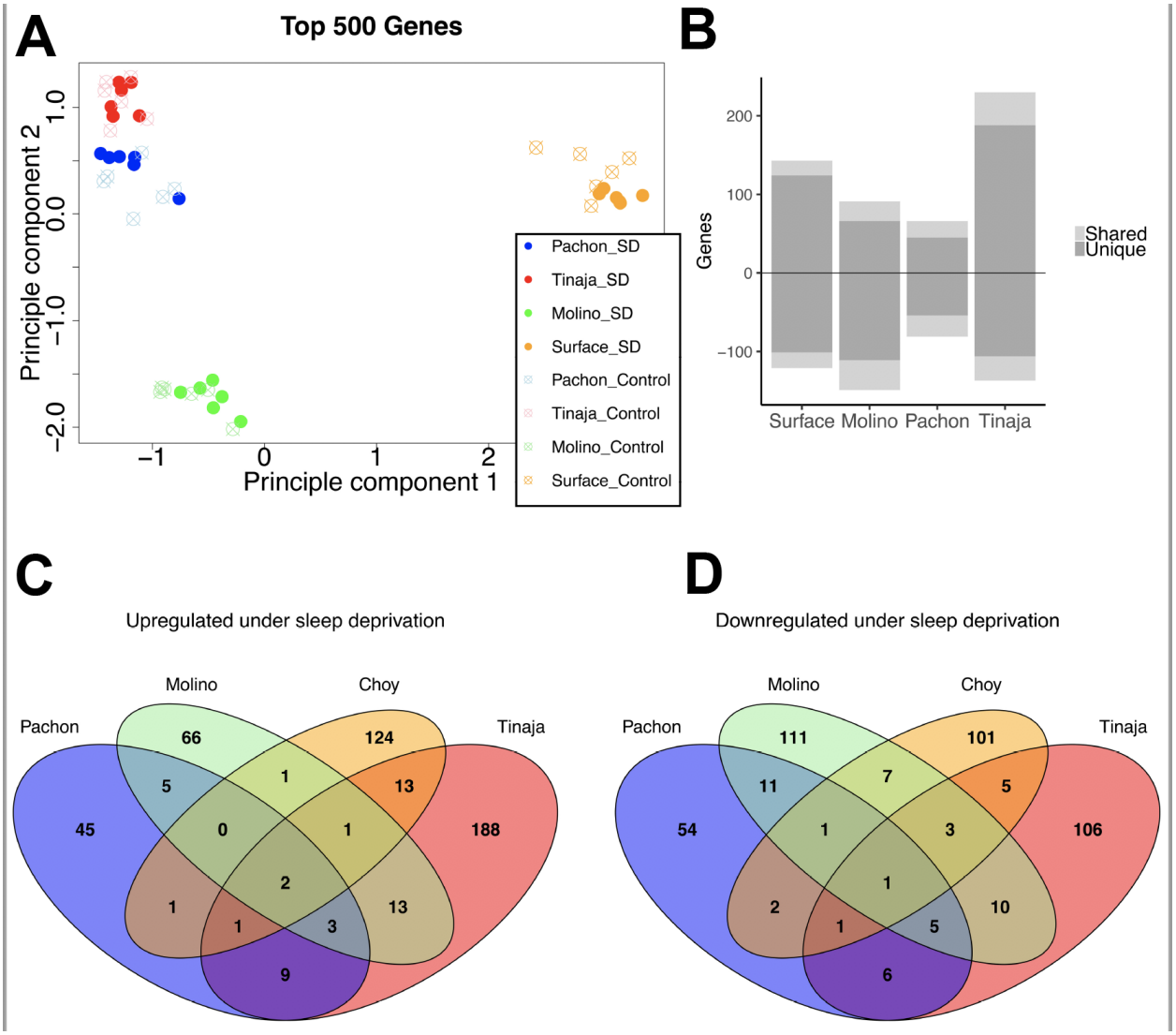

Table 1. Summary statistics of samples and sequencing. Standard deviation follows the mean number of raw reads (in millions). Upregulated and downregulated are given as population specific/total differentially expressed genes after correction for multiple testing. The percent that follows pertains to population specific genes.

Population - Treatment	N	Mean # Raw Reads	upregulated	downregulated
Surface - sleep deprived	6	16.90±0.52	124/143 (86.7%)	101/121 (83.5%)
Surface - control	6	17.18±1.84		
Molino cave - sleep deprived	6	17.91±1.07	66/91 (72.5%)	111/149 (74.5%)
Molino cave - control	6	16.48±1.75		
Pachón cave - sleep deprived	6	17.45±1.45	45/66 (68.2%)	54/81 (66.17)
Pachón cave - control	6	17.31±1.06		
Tinaja cave - sleep deprived	6	14.76±0.60	188/230 (81.7%)	106/137 (77.4%)
Tinaja cave - control	6	15.75±1.26		

606 **Data availability statement:**
607 All RNAseq reads are available on the SRA (Accession numbers given in Table S1).
608 Raw expression counts data are given as a supplementary file.
609
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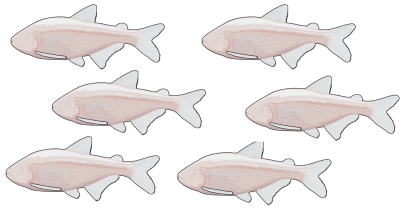

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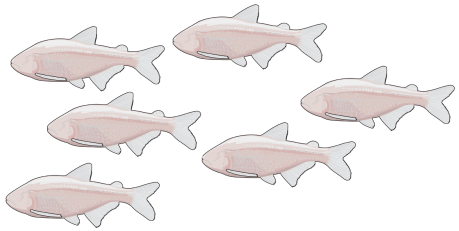

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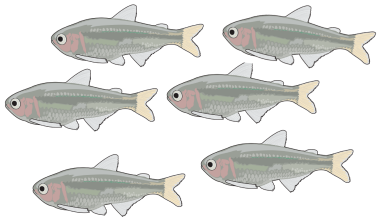

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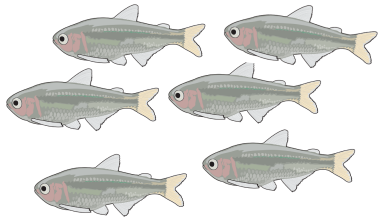


N = 6

Sleep deprivation
10 hrs during
normal "night"

10 hrs
normal "night"

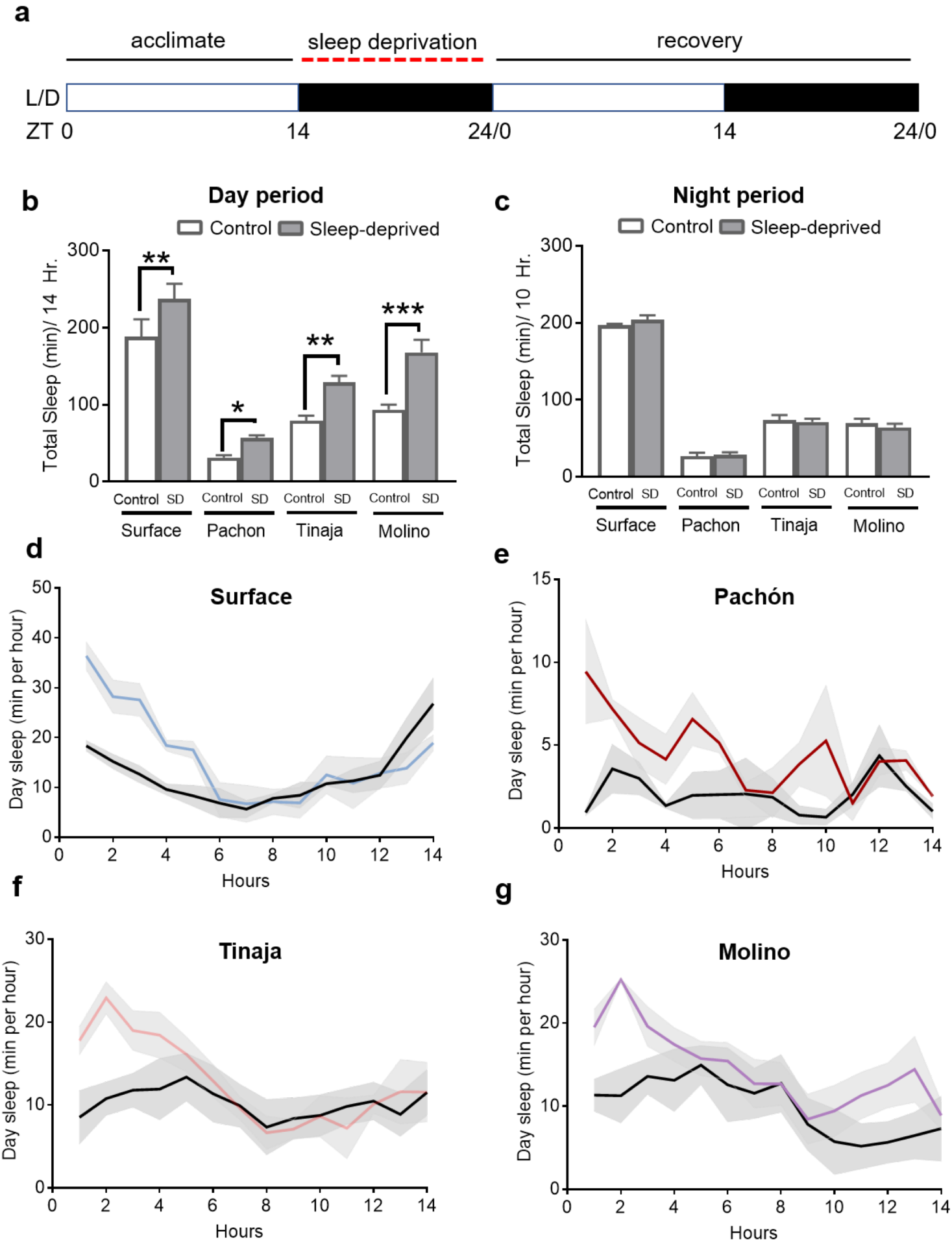
Sleep deprivation
10 hrs during
normal "night"

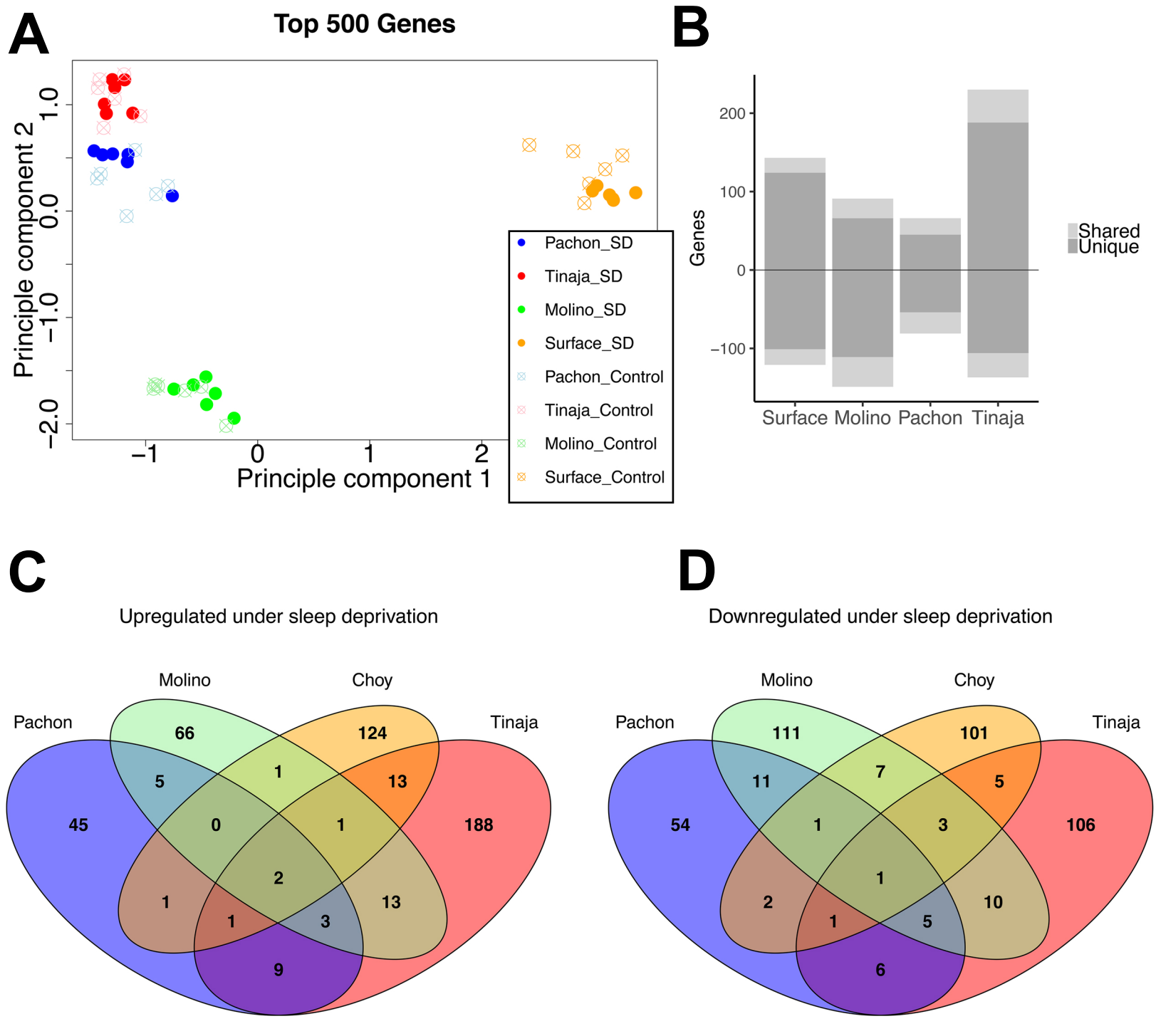
10 hrs
normal "night"

Flash freeze
at 6am

Randomize extraction date
Lane of sequencing

14.7-17.9 million reads/individual
17,187 genes included
post-filtering




A
Top 500 Genes
Principle component 2
Principle component 1
Pachon_SD
Tinaja_SD
Molino_SD
Surface_SD
Pachon_Control
Tinaja_Control
Molino_Control
Surface_Control
B
Genes
Shared
Unique
Surface
Molino
Pachon
Tinaja
C
Upregulated under sleep deprivation
Pachon
Molino
Choy
Tinaja
45
66
124
188
5
1
13
0
1
2
1
1
3
13
9
D
Downregulated under sleep deprivation
Pachon
Molino
Choy
Tinaja
54
111
101
106
11
7
5
1
3
2
1
10
1
5
6