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1 Unique transcriptional signatures of sleep loss across independently evolved cavefish
2 populations
3

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15 **Abstract**

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

39 Sleep is regulated by homeostatic drive and circadian gating (Archer & Oster, 2015;
40 Borbély, Daan, Wirz-Justice, & Deboer, 2016). Homeostatic sleep pressure increases throughout
41 the day for diurnal animals, while circadian drive aligns periods of rest and activity with light-
42 dark cycles (Archer & Oster, 2015; Borbély et al., 2016). Acute or chronic sleep loss induces
43 recovery sleep, suggesting the presence of a sleep homeostat. While the presence of a rebound in
44 response to sleep deprivation has been described in animals ranging from jellyfish to humans
45 (Anafi, Kayser, & Raizen, 2018; Keene & Duboue, 2018; Libourel et al., 2018; Nath et al.,
46 2017), surprisingly little is known about the molecular basis of this homeostat and even less is
47 known about resiliency of different genotypes to insufficient sleep (Diessler et al., 2018).
48 Further, it is unclear whether the genetic differences that underlie the robust differences in sleep
49 duration are linked to sleep homeostasis or resiliency to insufficient sleep (Keene & Duboue,
50 2018).

51 Understanding the functional relationship between sleep duration, homeostasis, and
52 resiliency to insufficient sleep would be aided by investigating the relationship between evolved
53 sleep loss and sleep homeostasis. The Mexican tetra, *Astyanax mexicanus*, has emerged as a
54 leading model for investigating the evolution of sleep (Jaggard et al., 2017; Jaggard et al., 2018;
55 Keene, Yoshizawa, & McGaugh, 2015). *Astyanax mexicanus* are a single species consisting of
56 eyed surface fish that inhabit the rivers of northeast Mexico and Texas and at least 30
57 populations of conspecific blind, cave-dwelling fish (Bradic et al., 2012; Coghill et al., 2014;
58 Espinasa, Rivas-Manzano, & Pérez, 2001; Herman et al., 2018). Several independent origins of
59 the cave phenotype arose within the past ~200k years and effective population sizes of
60 independent caves suggest that selection may be an efficient force in shaping cave-derived traits
61 (Bradic et al., 2012; Coghill et al., 2014; Herman et al., 2018). Sleep is reduced compared to
62 surface fish in five cave populations studied to date, suggesting the evolutionary convergence on
63 sleep loss (Duboué, Keene, & Borowsky, 2011; Jaggard et al., 2018; Yoshizawa et al., 2015).
64 Further, distinct neural mechanisms lead to sleep loss between populations, indicating sleep loss
65 evolved independently in different cavefish populations (Jaggard et al., 2017; Jaggard et al.,
66 2018).

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

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69 resiliency to insufficient sleep is unclear. Previously, it was reported that one cavefish population
70 displayed a rebound following deprivation and the other tested populations approached
71 significance (Duboué et al., 2011). Given the robust evolutionary changes in sleep duration
72 across populations, it is not clear whether rebound sleep in cavefish is dependent on shared or
73 divergent molecular mechanisms from surface fish. Here, we employ a new method of acute
74 sleep deprivation and find surface fish and three cavefish populations display robust sleep
75 rebound following a single night of deprivation.
76 A recently sequenced genome in *A. mexicanus* allows for genome-wide analysis of
77 context-dependent changes in gene-expression (McGaugh et al., 2014). While transcriptomic
78 approaches have been used to identify developmentally-specified changes in gene expression in
79 *A. mexicanus* (Gross, Furterer, Carlson, & Stahl, 2013; Hinaux et al., 2013; Stahl & Gross), they
80 have not been applied to identify novel regulators of behavior. The power of comparing between
81 independent populations of cavefish with robust differences in sleep behavior, allows for
82 investigation of mechanisms underlying sleep homeostasis as well as physiological and
83 metabolic responses to sleep deprivation.
84 Here we compare the behavioral and molecular signatures of sleep deprivation in surface
85 fish and three populations of cavefish. We find that recovery sleep is maintained in cavefish
86 populations despite robust evolutionary loss of sleep duration in undisturbed conditions.
87 Transcriptome analysis revealed unique signatures of sleep deprivation suggesting distinct
88 responses to insufficient sleep across all populations.
89
90 **Methods**
91 Animal Husbandry
92 Animal husbandry was carried out as previously described (Borowsky, 2008), and all protocols
93 and procedures for this experiment were approved by the IACUC Florida Atlantic University
94 (Protocols A16-04 and A17-32). Fish were housed in the Florida Atlantic University core
95 facilities at 23 ± 1°C constant water temperature throughout rearing for behavior experiments
96 (Borowsky, 2008). Lights were kept on a 14:10 hr light-dark cycle that remained constant
97 throughout the animal's lifetime. Light intensity was kept between 25–40 Lux for both rearing
98 and behavior experiments. All fry were housed in 250 mL pyrex dishes (0-10 dpf, days post
99 fertilization) or 1L tanks (11-30 dpf) and fed *Artemia* twice daily.

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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

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

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

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

152 For the Molino and Pachón populations, control fish were from a spawning of the same
153 parental fish as sleep deprived fish and were exactly 30dpf when sampled, however they were
154 sampled from a clutch that was spawned approximately a week after the sleep deprived treatment
155 fish. We sampled control fish on the same day of as the sleep deprivation fish, but due to a
156 technical error, we could only sequence one control individual from Molino and Pachón from the
157 same sampling day as the sleep deprived fish. We used this single control sample from Molino
158 and Pachón to determine whether collection days drove a substantial amount of variation. MDS
159 plots revealed that there was little difference between these two sampling times (Figure S1).
160

161 RNA extraction, library preparation and sequencing

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162 Protocols for RNA extraction, library preparation, and sequencing were performed as
163 previously described (Passow et al., 2019). To isolate RNA, whole 30dpf fry (~ 30 mg of tissue)
164 were homogenized and then lysed. Total RNA was extracted using the Qiagen RNAeasy Plus
165 Mini Kit (Qiagen) and then quantified (ng/uL) using NanoDrop Spectrophotometer (Thermo
166 Fisher Scientific), Ribogreen assay (Thermo Fisher Scientific), and Bioanalyzer RNA 6000 Nano
167 assay (Agilent). All individuals were extracted within a week by same researcher. The extraction
168 batch was randomized across populations and treatments (Table S1).
169 All cDNA libraries were constructed at the University of Minnesota Genomics Center on
170 the same day in the same batch, and 400ng of total RNA was used for mRNA isolation and
171 cDNA synthesis. Strand-specific cDNA libraries were then constructed using TruSeq Nano
172 Stranded RNA kit (Illumina), following manufacturer protocol. Before libraries were pooled for
173 sequencing, library quality was assessed using Agilent DNA 1000 assay on a Bioanalyzer. Only
174 two samples exhibited RIN scores < 9 from the extracted total RNA (Table S1).
175 To minimize sequencing lane effects, barcoded libraries were pooled with treatment and
176 population spread evenly between lanes. Samples were sequenced across multiple lanes of an
177 Illumina HiSeq 2500 to produce 125-bp paired-end reads at University of Minnesota Genomics
178 Center (Table S1). Little variation was explained by extraction batch or lane of sequencing
179 (Passow et al., 2019). All sequence data were deposited in the short read archive (Table S1).
180
181 Gene expression quantification
182 The pipelines used to filter and quantify patterns of differential expression were similar to
183 previous published work (Passow et al., 2019), with minor adjustments. In brief, the raw RNA-
184 seq reads were quality checked using Fastqc (Andrews, 2014) and then trimmed to remove
185 adapters and poor quality reads using the program Trimmomatic v0.33 (Bolger, Lohse, &
186 Usadel, 2014). Trimmed reads were then mapped against the *Astyanax mexicanus* Pachón
187 reference genome (version 1.0.2; GenBank Accession Number: GCA_000372685.1; McGaugh et
188 al., 2014) using the splice-aware mapper STAR (Dobin et al., 2013). Stringtie v1.3.3d (Pertea et
189 al., 2016; Pertea et al., 2015) was used to quantify the number of reads mapped to each gene in
190 the reference *A. mexicanus* genome annotation. Finally, to generate the counts matrix, we used
191 the python script provided with Stringtie (prepDE.py; Pertea et al., 2016). Annotations were

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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

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223 Selection analyses

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

234 Our procedures for outlier analyses have been described (Yoshizawa et al., 2018), but we
235 briefly recap here. We used hapFLK v1.3 https://forge-dga.jouy.inra.fr/projects/hapflk (Fariello
236 et al., 2013) for genome-wide estimation of the hapFLK statistic of across all 44 *Astyanax*
237 *mexicanus* samples and two *Astyanax aeneus* samples. HapFLK accounts for hierarchical
238 population structure by building local ancestry trees and detects changes in haplotype
239 frequencies which exceed what is expected for neutral evolution (Fariello et al., 2013). HapFLK
240 outperformed many other statistics (Schlamp et al., 2016) and may be robust to bottlenecks and
241 migration. We augment this measure with metrics of F_{ST}, π, and d_{XY}. We also required that π that
242 was not excessively low in the two surface populations, Rascon and/or Río Choy, (rank of
243 greater than 500 for dense ranking of genes or lowest ~2.5% of diversity values in the genome)
244 to protect, in part, against calling outliers from inflated relative measures of divergence that were
245 driven by low diversity due to low recombination or sweeps in the surface population. As we are
246 interested in identifying genes under strong selective sweeps in the cave populations, we did not
247 want to exclude genes with low diversity in cave populations. F_{ST} is more sensitive to changes in
248 allele frequency than absolute measures such as d_{XY} (Cruickshank & Hahn, 2014).
249

250 **Results**

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

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253 shaking shown to elicit sleep rebound. Fry were sampled for behavioral sleep rebound and RNA-
254 seq at 30dpf.
255
256 Sleep homeostasis behavioral assays
257 To quantify sleep homeostasis across *A. mexicanus* populations, we sleep deprived fish
258 throughout the night period (ZT14-24) and compared sleep the following day to undisturbed
259 controls (Figure 2A). Surface fish, as well as Pachón, Molino, and Tinaja cavefish displayed a
260 significant increase in daytime sleep following deprivation suggesting sleep homeostasis is intact
261 in all three cavefish populations (Figure 2B, D-G). When sleep measurements were extended
262 into the night following deprivation (ZT14-24), total sleep duration did not differ between
263 control and sleep deprived fish across all four populations tested (Figure 2C, D-G), suggesting
264 the recovery sleep during the day was sufficient to return sleep drive to levels in undisturbed
265 fish. Taken together, these findings reveal sleep homeostasis is intact across three populations of
266 cavefish, despite exhibiting robustly diminished basal sleep levels.
267
268 Mapping statistics
269 On average we sequenced 16.72 million reads per sample (median = 16.77 million, min = 13.36,
270 max = 19.88) from whole, individuals at 30 dpf (Table 1, Table S1, Supplemental materials).
271 Pachón cavefish exhibited slightly higher percentage of reads mapped compared to all other
272 populations, as would be expected given reference sequence bias. Total yield of reads were not
273 significantly different between treatments (Wilcoxon test W = 252.5, p-value = 0.4705). On
274 average, 88.96% reads mapped per sample to the *Astyanax mexicanus* genome (median = 88.9%,
275 range 85.06% - 91.59%). Filtering of the gene counts matrix to include only transcripts with
276 greater than two cpm and three or more individuals with counts data resulted in 17,187 genes
277 used for differential expression analysis and clustering analysis, except where otherwise noted.
278 Libraries were normalized by size using calcNormFactors (Figure S2), and samples did not
279 exhibit clustering due to lane of sequencing or extraction batch (Figure S3, S4).
280
281 General patterns in expression response to sleep deprivation
282 After correcting for multiple testing, the number of significantly differentially expressed genes
283 between sleep deprived and control groups is < 2.1% of the 17,187 genes used for differential

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284 expression analyses in all populations (Table 1). Multidimensional scaling analysis showed that
285 the major axis of differentiation among the top 500 genes was ecotype (Figure 3) and explained
286 33% of the variation, suggesting that expression profiles of cave populations are more similar to
287 each other than to surface fish. The second principal component accounted for 12% of the
288 variation in the data and separated the caves into two clusters containing 1) Pachón-Tinaja and 2)
289 Molino, which represent two separate lineages of cavefish (Herman et al., 2018). Thus, ecotype
290 and population (and not sleep deprivation treatment) drove the majority of variation in the
291 complete dataset. Since population is such a strong driver of expression, to isolate the effect of
292 sleep deprivation on expression profiles, we separated the populations into individual MDS plots
293 (Figure S5, S6, S7, S8).
294 Across population-specific MDS plots, sleep deprivation treatment accounted for 12-16%
295 of the variation in the dataset. However, individual populations exhibited different signatures.
296 Most notably, Pachón cave population exhibits little differentiation between sleep deprived and
297 control samples (Figure S5) compared to the other populations (Figure S6, S7, S8).
298
299 Divergent responses across cave and surface populations in response to sleep deprivation
300 Despite surface fish exhibiting greater than 3-fold longer total sleep than cavefish at this stage of
301 development (Duboué et al., 2011and Figure 1), the 10-hour sleep deprivation treatment did not
302 result in a significant increase in the total number of differentially expressed genes in surface fish
303 compared to any of the cave populations. Surface fish exhibited ~1.6 and ~2.2 fold the number of
304 upregulated genes in response to sleep deprivation than Molino and Pachón cavefish,
305 respectively (Table 1). Surface fish also experienced ~1.5 fold the number of downregulated
306 genes than Pachón cavefish (Table 1). However, Tinaja cavefish exhibited a greater total number
307 of upregulated genes than the three other populations (Table 1). Thus, we did not observe a direct
308 pattern that evolved sleep loss in cave populations results in fewer differentially expressed genes
309 in response to sleep deprivation, fortifying the behavioral findings that sleep homeostasis is
310 largely intact in cavefish population.
311 While surface fish did not exhibit more exacerbated transcriptional responses to sleep
312 deprivation than cavefish, the surface population exhibited the highest number of uniquely
313 differentially expressed genes than any other population (83.5-86.7%, Table 1), suggesting
314 surface-specific responses to sleep deprivation. More cave-specific overlap in differentially

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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.,

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346 2018). While this gene is not annotated in any fish genome on Ensembl, it is notable that
347 cytoskeleton-related processes are enriched in genes upregulated in response to sleep deprivation
348 in Tinaja cavefish. Downregulated genes for cave populations were associated with winged
349 helix/forkhead transcription factors in Molino ($p = 0.054$, after FDR correction) and nitrogen
350 compound transport in Tinaja. In the surface population, upregulated genes are significantly
351 enriched for transmembrane transport functions of a variety of molecules, but downregulated
352 genes exhibit no significant functional enrichment. The different GO categories for each
353 population support the notion that sleep deprivation evokes variable intraspecific physiological
354 responses across different *A. mexicanus* populations.
355
356 <u>Selection analyses</u>
357 Several differentially expressed genes also exhibit signatures of positive selection (Table S14).
358 Notably, *aif1l* (*allograft inflammatory factor 1 like*) is downregulated in Molino and Pachón
359 cavefish in response to sleep deprivation and exhibited population genetic parameters suggestive
360 of a recent selective sweep in both populations (e.g., significant with HapFLK analyses, in the
361 top 5% of F_{ST} values across the genome between surface and Molino populations, in the top 10%
362 of F_{ST} values across the genome between surface and Pachón populations, and one of the least
363 diverse genes across the genome in both cave populations). Knockout mice of *aif1l* exhibited
364 deformities in endocrine/exocrine, hematopoietic, immune, integument, and liver/biliary systems
365 (Dickinson et al., 2016), suggesting a pleiotropic link between extended wakefulness and liver
366 and immune function in cavefish. Next, *slc17a8* is downregulated in Tinaja cavefish in response
367 to sleep deprivation and is involved in equilibrioception, neuromast hair cells, neuron-neuron
368 synaptic transmission, posterior lateral line neuromast hair cell development, startle response,
369 and vestibular reflex (Obholzer et al., 2008). Population genetic parameters of *slc17a8* suggest a
370 recent selective sweep in Tinaja (significant with HapFLK, in the top 5% F_{ST} outliers between
371 Tinaja cave and Rascon surface, while being among the lowest diversity genes in Tinaja). The
372 down regulation of lateral line components in response to sleep deprivation is especially
373 interesting since sleep duration in at least one cavefish population is restored with the ablation of
374 the lateral line (Jaggard et al., 2017). Our results suggest that dampening signals from the lateral
375 line may be a response to sleep pressure. Interestingly, Pachón cavefish exhibited significant
376 upregulation of the retinal photoreceptor-associated gene *phosphodiesterase 6G* (*pde6gb*) which

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377 is highly enriched in the pineal gland relative to the brain of larval and adult zebrafish (Toyama
378 et al., 2009), suggesting *pde6gb* may play a role in circadian physiology. This gene exhibited
379 metrics of a recent selective sweep in Pachón (e.g. significant with HapFLK analysis, in the
380 largest 20% Dxy and largest 5% Fst for Pachón-surface comparisons and exhibits very low
381 diversity in Pachón). The three cases highlighted here suggest that selection in cave populations
382 may have shaped liver, lateral line, and photoreceptor functioning, and these traits pleiotropically
383 impacted sleep phenotypes or vice versa.
384
385 **Discussion**
386 While sleep duration is widely studied across animal species, sleep homeostasis and
387 baseline sleep phenotypes are likely functionally distinct (Anafi et al., 2018; Joiner, 2016; Keene
388 & Duboue, 2018). A central question is whether the truncated sleep duration that has evolved in
389 cavefish (Duboué et al., 2011; Jaggard et al., 2018) also impacted sleep homeostasis or whether
390 these processes are independently regulated. Here we implemented an acute sleep deprivation
391 treatment to examine the behavioral and molecular effects in surface fish and three
392 independently-evolved cavefish populations. Our behavioral analysis confirms previous findings
393 that sleep homeostasis is intact across cavefish populations (Duboué et al., 2011), supporting the
394 notion that baseline sleep phenotypes are regulated by separate processes from sleep homeostasis
395 (Allada, Cirelli, & Sehgal, 2017). Sleep significantly deprivation impacted expression of only a
396 few hundred genes across populations, and different cavefish populations and surface fish exhibit
397 discordant gene expression responses to the same sleep deprivation treatment. Thus, our results
398 demonstrate intraspecific variation in molecular mechanisms of sleep pressure in response to
399 sleep deprivation.
400 Sleep deprivation is an effective way to induce sleep rebound and results in a host of
401 pathophysiological and cognitive deficits (Cappuccio, D'elia, Strazzullo, & Miller, 2010;
402 Cedernaes et al., 2018; Leproult, Holmbäck, & Van Cauter, 2014). Our analysis comparing gene
403 expression in control and sleep deprived fish revealed differential gene expression in only a few
404 hundred genes in response to sleep deprivation across all populations. However, we observed
405 three genes that were differentially expressed across all cave and surface populations, indicating
406 they are part of a generalized response to sleep deprivation: *heat shock protein alpha-crystallin-*
407 *related, b11* (*hspb11*) and *neutral cholesterol ester hydrolase 1a* (*nceh1a*) were upregulated and

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408 transcription factor *E74-like factor 3* (*elf3*) was downregulated in response to sleep deprivation.
409 Heat shock proteins and other molecular chaperones are often upregulated after sleep deprivation
410 as an indicator of cellular stress, suggesting this is an evolutionarily conserved response to sleep
411 deprivation (Allada et al., 2017; Cedernaes et al., 2018; Mackiewicz et al., 2009; Uyhelji et al.,
412 2018). N*eutral cholesterol ester hydrolase 1a* (*nceh1a*) promotes adipogenesis (Homan, Kim,
413 Cardia, & Saghatelian, 2011), and is strongly implicated in atherosclerotic lesions (Igarashi et al.,
414 2010; Okazaki et al., 2008), indicating that *nceh1a* may be a candidate for mediating the well-
415 documented relationships between short sleep duration and advancement of atherosclerosis and
416 weight gain (Cedernaes et al., 2018; Davies et al., 2014; Levy et al., 2012; Nakazaki et al., 2012).
417 *E74-like factor 3* (*elf3*) is the only gene significantly downregulated in response to sleep
418 deprivation in all populations. Interestingly, this gene is involved in inflammatory response
419 (Conde et al., 2016), epithelial cell differentiation (Kwon et al., 2009), and is a cancer gatekeeper
420 (Gajulapalli et al., 2016; Shatnawi et al., 2014; Yachida et al., 2016; Yeung et al., 2017),
421 suggesting a molecular link between sleep disruption and increased cancer risk (Blask, 2009;
422 Haus & Smolensky, 2013). Together, these genes may be conserved factors that are regulated in
423 accord with sleep drive and deprivation across *A. mexicanus* populations.
424 All genes that were not differentially expressed in the surface population, but were
425 significantly upregulated across all cave populations were linked to sleep or circadian function.
426 *Carnitine palmitoyltransferase 1B* (*cpt1b*) variants are associated with narcolepsy in humans and
427 upregulated *cpt1b* may increase hypocretin activity (Han et al., 2012; Miyagawa et al., 2008),
428 consistent with previous work demonstrating that increased hypocretin is highly associated with
429 short sleep duration in cavefish (Jaggard et al., 2017; Jaggard et al., 2018). *aarF domain*
430 *containing kinase 5* (*adck5*), was upregulated across all cave populations in response to sleep
431 deprivation and human variants are associated with insomnia symptoms (Lane et al., 2017).
432 *Telethonin* (*tcap*), a component of cardiac sarcomeres, is regulated by circadian components
433 CLOCK and BMAL1 (Podobed, Alibhai, Chow, & Martino, 2014), supporting the close
434 relationship between sleep homeostasis and circadian cycles suggested by other work (Allada et
435 al., 2017; Borbély et al., 2016). In contrast, none of the genes that were significantly
436 downregulated in response to sleep deprivation across all caves have direct links to sleep or
437 circadian function (e.g. *gamma-glutamyl hydrolase-like, ETS homologous factor*, or *cathepsin*
438 *K*), though *transmembrane and coiled-coil domain family 1* (*TMCC1*) may potentially be

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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

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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

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500 conserved signaling molecules in sleep regulation across the animal phylogeny. These include
501 *aanat2*, an important enzyme for the production of melatonin, gaba receptors (*gabarapa*,
502 *gabarapl2*), brain derived neurotrophic factor (*bdnf*), adenosine receptors (*adora1a*, *adora1b,*
503 *adora2a*, *adora2b*), adenosine deaminase (*ada*, *adar*), adenosine kinase (*adka*, *adkb*) (Holst &
504 Landolt, 2015), NMDA receptors (e.g., *grin* paralogs, *nsmfa*) (Liu, Liu, Tabuchi, & Wu, 2016),
505 *flotillin* (*flot1*) (Mackiewicz et al., 2007) and dopamine receptors and transporters (*drd1b*, *drd4a*,
506 *drd4b*, *slc6a3*). Likewise, key clock genes (*clockb*, *per1a*, *per1b, per2*, *per3*, *arntl1a*, *arntl1b*,
507 *cry1aa*, *cry1ab, cry1ba*, *cry1bb, cry4*, *roraa*, *rorab*, *rorc*), which are often impacted in sleep
508 deprivation studies (Allada et al., 2017; Archer et al., 2014; Archer & Oster, 2015; Borbély et al.,
509 2016; Franken, 2013; Möller-Levet et al., 2013; Uyhelji et al., 2018), are all not differentially
510 expressed for any tetra population. Together, this suggests that genes regulating sleep duration
511 and circadian function under standard conditions are largely unaffected by a single night of sleep
512 deprivation in tetras.

513 Several considerations must be taken into account in evaluating our study. First, our
514 study examined the effect of a single night of sleep deprivation in fish housed on a standard
515 14:10 light cycle. While the results suggest relatively limited changes in the number of
516 differentially expressed genes, it is consistent with studies examining the effects of acute sleep
517 deprivation in other animals. For example, a similar number of genes had altered expression
518 levels in humans after various levels of sleep deprivation (Aho et al., 2013; Cedernaes et al.,
519 2018; Pellegrino et al., 2012). We predict that longer-term sleep deprivation for days or chronic
520 insufficient sleep over a number of days may result in more robust changes in gene expression,
521 however, these protocols would also be likely to induced generalized stress (Pellegrino et al.,
522 2012).

523 Second, our study employed whole-body sampling for RNA-seq from mRNA transcripts.
524 Tissue specific differences are documented to result from sleep deprivation (Cedernaes et al.,
525 2018) and may obscure signal from specific genes (Diessler et al., 2018). For example, *per2*
526 expression increases in sleep deprived mice and remains elevated for varied amounts of time
527 depending on the tissue (Curie, Maret, Emmenegger, & Franken, 2015). At 30dpf, brain
528 dissection is technically challenging and would likely require pooling across samples. Further,
529 precise dissection of tissue takes time and would result in the samples collected last being sleep
530 deprived for longer than the samples collected first.

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531 To our knowledge, these findings are the first genome-wide analysis of sleep deprivation
532 induced changes in fish. In zebrafish, sleep deprivation robustly impacts cellular processes and
533 behavior (Aho et al., 2017; Elbaz et al., 2017; Pinheiro-da-Silva, Silva, Nogueira, & Luchiari,
534 2017; Zada et al., 2019), but the effects on large-scale changes in gene expression have not been
535 investigated. Both zebrafish and *A. mexicanus* provide robust models for the identification of
536 genetic and pharmacological regulators of sleep (Duboué et al., 2012; Jaggard et al., 2018;
537 Prober, 2018; Rihel, Prober, & Schier, 2010), suggesting these models can be used to investigate
538 the genetic architecture associated with sleep loss. Our findings, that sleep deprivation induces
539 different molecular signatures in each of the four *A. mexicanus* populations tested, raises the
540 possibility that the response to sleep pressure is highly heterogenous across individuals of the
541 same species. The application of recently developed gene-editing approaches in *A. mexicanus*
542 (Ma et al., 2018; Stahl et al., 2019) combined with the behavioral assay described here may
543 allow for functional validation of these genes and identification of novel regulators of sleep
544 homeostasis.
545
546 **Acknowledgements**
547 We thank the University of Minnesota Genomics Center for their guidance and performing the
548 cDNA library preparations and Illumina HiSeq 2500 sequencing. The Minnesota
549 Supercomputing Institute (MSI) at the University of Minnesota provided resources that
550 contributed to the research results reported within this paper. Funding was supported by NIH
551 (1R01GM127872-01 to SEM and ACK) and NSF award IOS 165674 to ACK, and a US-Israel
552 BSF award to ACK. CNP was supported by Grand Challenges in Biology Postdoctoral Program
553 at the University of Minnesota College of Biological Sciences. Institutional Animal Care and
554 Use Committee at Florida Atlantic University (Protocol #A15-32).
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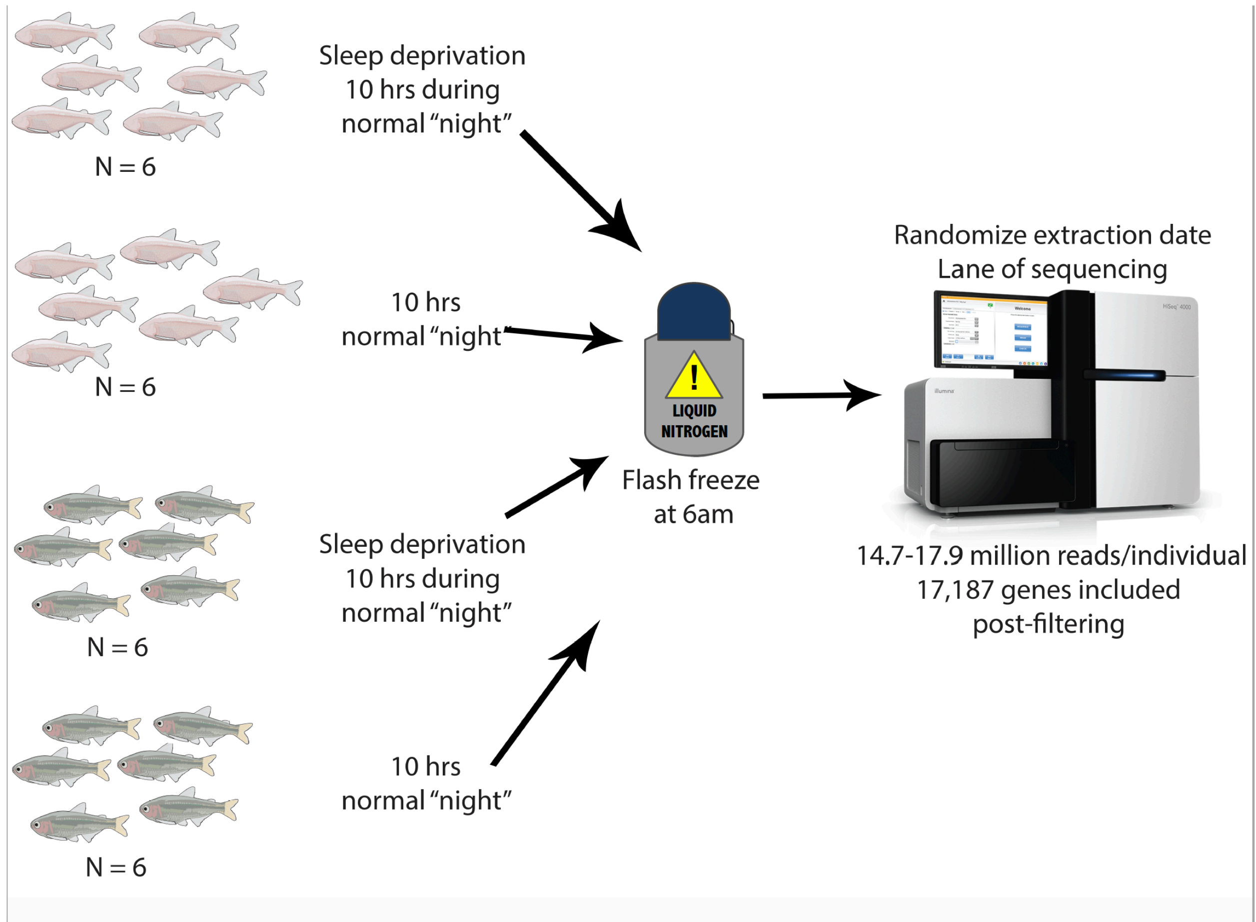
557 **Figure 1.** Sampling schematic. We sampled three cave populations: Tinaja, Pachón, and Molino
558 and one surface population at 30 days post fertilization. Fish were sleep deprived for 10hrs
559 during their normal dark period through intermittent shaking.
560



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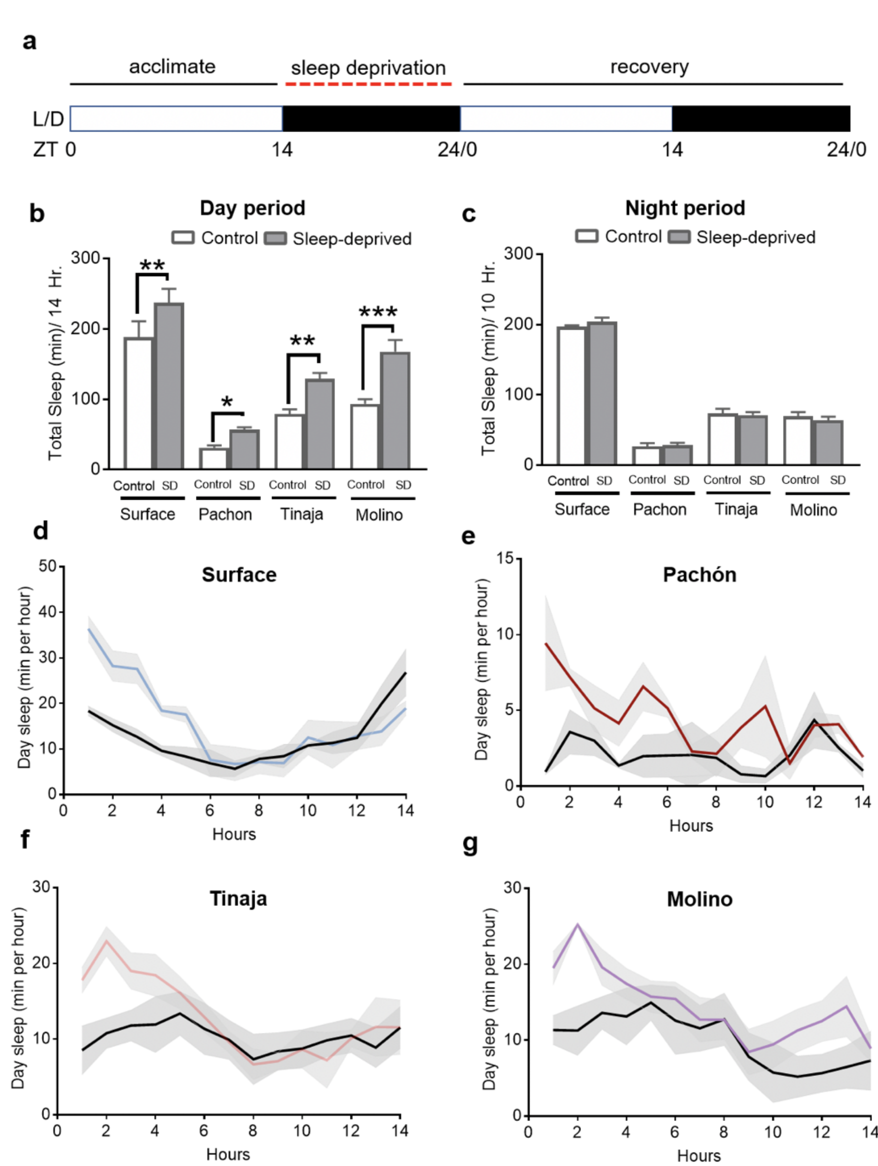
563 **Figure 2. A)** Following 14hrs of acclimation, fish were sleep deprived for 10 hours (red line)
564 during the night and behavior was measured over the following 24 hrs. **B)** All four groups of
565 sleep deprived fish (grey) slept significantly more than undisturbed controls (white) during the
566 14hrs of daytime sleep following deprivation due to longer sleep bouts. **C)** No differences in
567 sleep duration were detected between group during the 10 hrs of night following deprivation.
568 Sleep profiles for **D)** surface, **E)** Pachón, **F)** Tinaja, and **G)** Molino over the day following
569 deprivation. Color lines denote sleep derived fish, black lines represent undisturbed controls.
570 Grey represents standard error of the mean.
571



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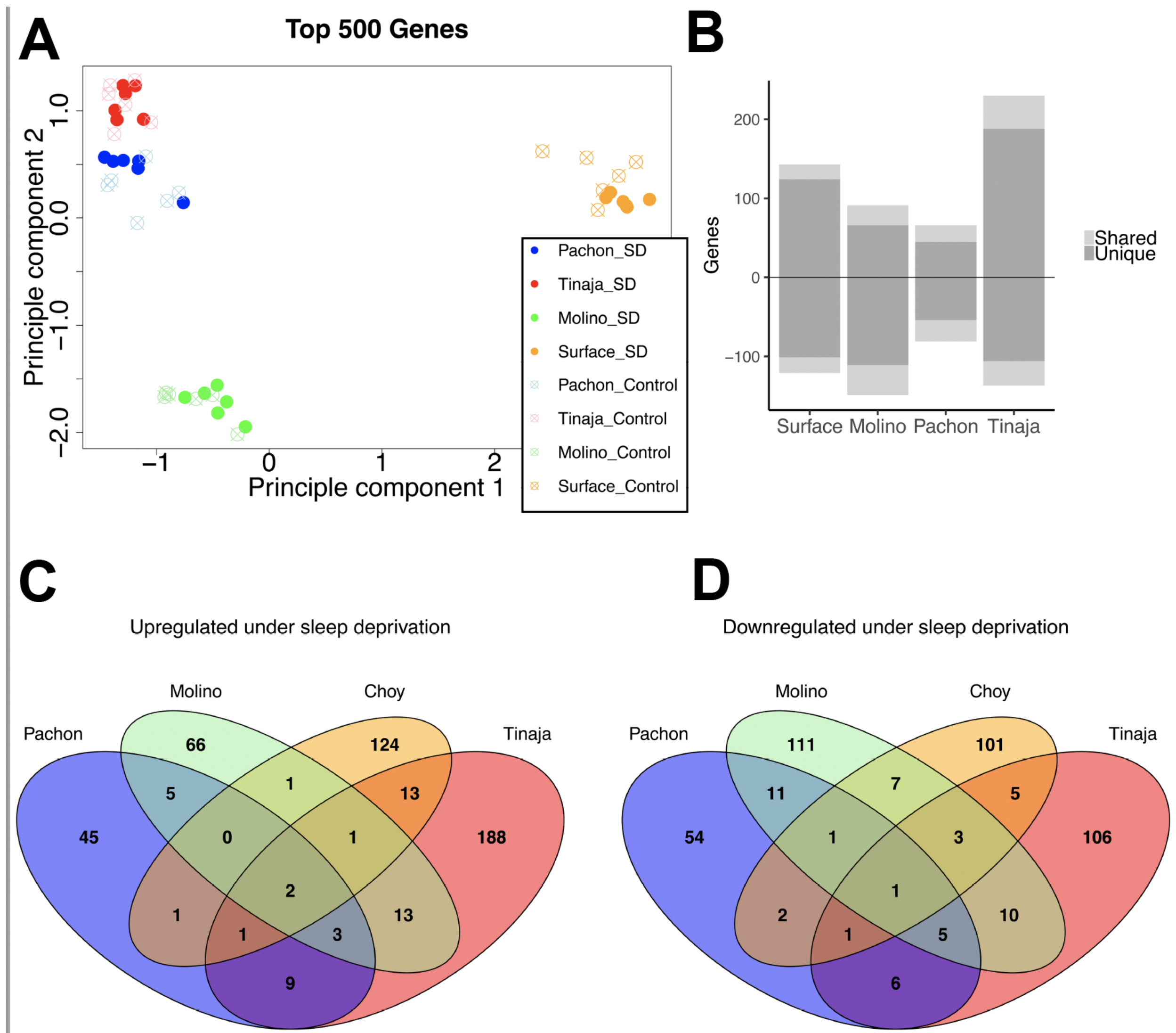
572 **Figure 3. A)** MDS plot of 500 Top genes between control and sleep deprivation treatments the
573 four populations normalized for library size. PC1 clearly demarcates population of origin. **B)**
574 Number of genes differentially expressed between control and sleep deprived treatments. Above
575 zero is upregulated in sleep deprived samples and below zero is downregulated in sleep deprived
576 samples. Light grey = shared with at least one other population, Dark grey = not shared among
577 other populations. Overall few genes are differentially expressed in response to sleep deprivation
578 and differentially expressed genes are not shared among populations. **C)** Venn-diagram of
579 overlap in differentially upregulated genes in the sleep deprivation treatment between all four
580 populations. **D)** Venn-diagram of overlap in differentially downregulated genes in the sleep
581 deprivation treatment across all four populations.
582



583
584
585
586

587 **Table 1. Summary statistics of samples and sequencing.** Standard deviation follows the mean number of raw reads (in millions).
588 Upregulated and downregulated are given as population specific/total differentially expressed genes after correction for multiple
589 testing. The percent that follows pertains to population specific genes.
590

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		

591–605

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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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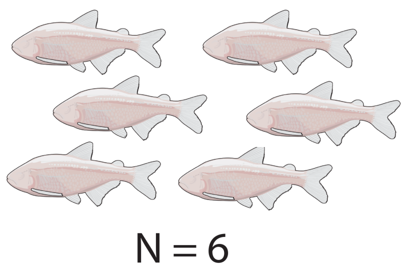
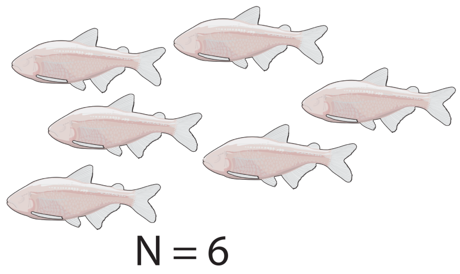


Sleep deprivation
10 hrs during
normal "night"

N = 6



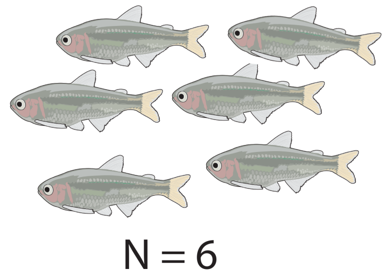
10 hrs
normal "night"

N = 6

Flash freeze
at 6am

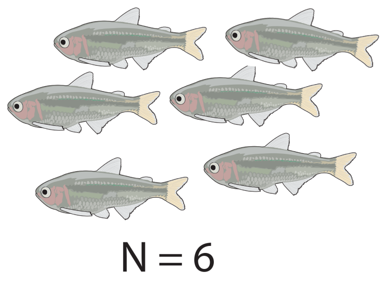
Randomize extraction date
Lane of sequencing

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



Sleep deprivation
10 hrs during
normal "night"

N = 6



10 hrs
normal "night"

N = 6

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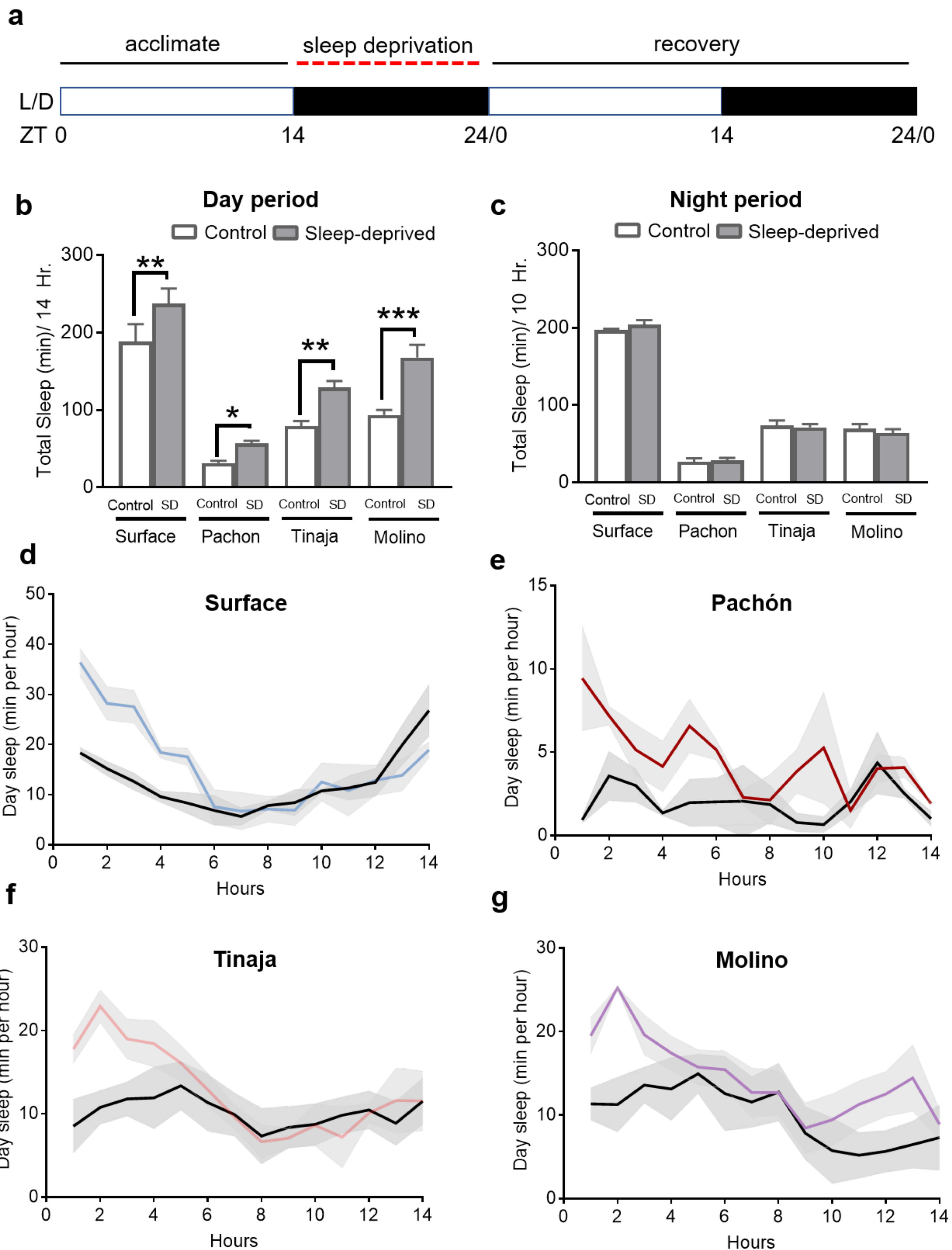
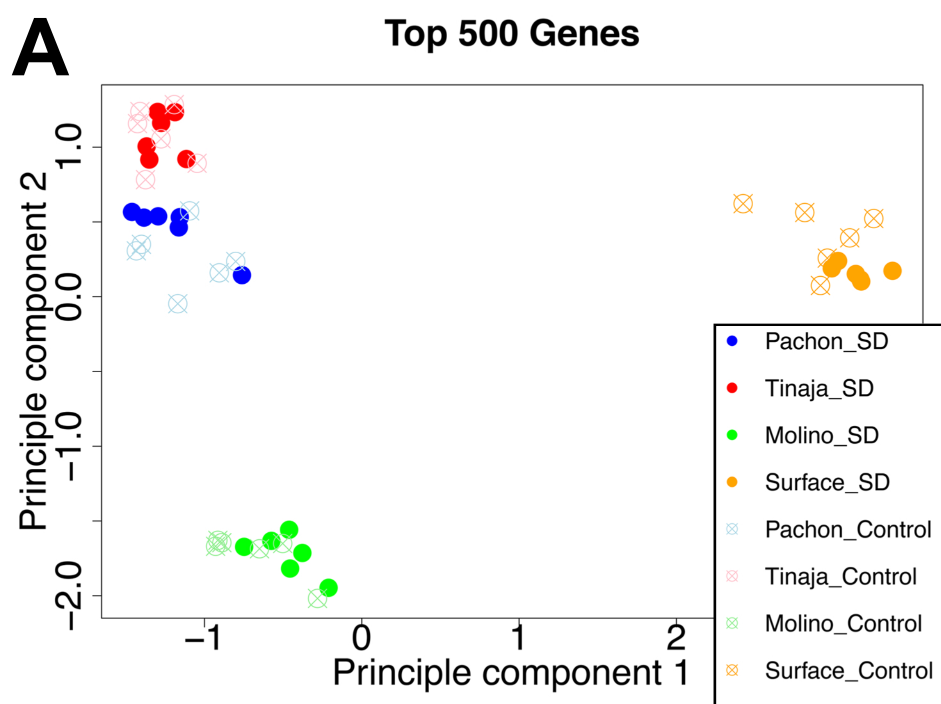
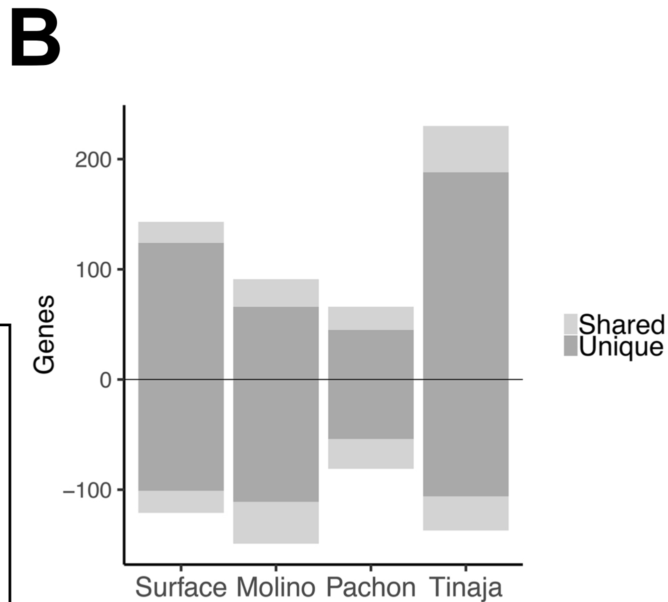



A
Top 500 Genes
Principle component 2
Principle component 1
1.0
0.0
−1.0
−2.0
−1
0
1
2
Pachon_SD
Tinaja_SD
Molino_SD
Surface_SD
Pachon_Control
Tinaja_Control
Molino_Control
Surface_Control


B
Genes
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−100
Surface
Molino
Pachon
Tinaja
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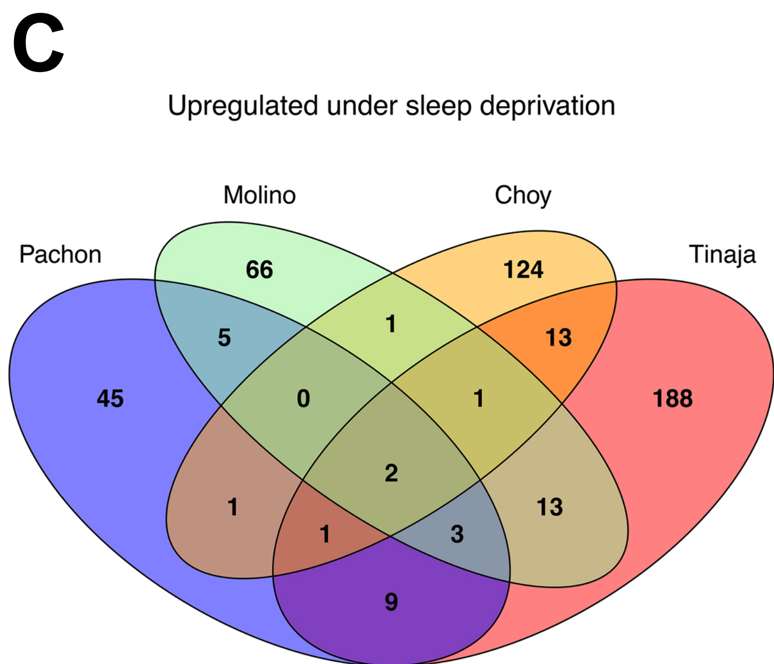
Unique


C
Upregulated under sleep deprivation
Pachon
Molino
Choy
Tinaja
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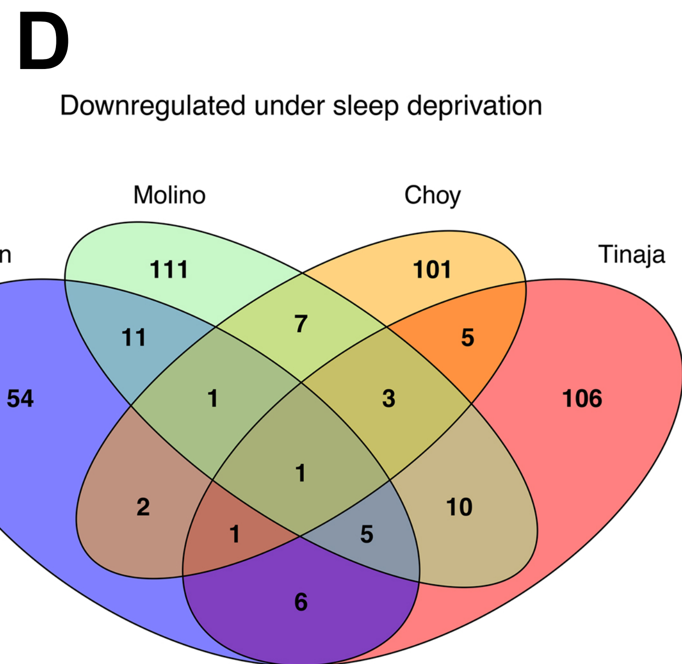
D
Downregulated under sleep deprivation
Pachon
Molino
Choy
Tinaja
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