Phenotypic plasticity as a mechanism of cave colonization and adaptation

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ABSTRACT

A widely accepted model for the evolution of cave animals posits colonization by surface ancestors followed by the acquisition of adaptations over many generations. However, the speed of cave adaptation in some species suggests mechanisms operating over shorter timescales. To address these mechanisms, we used *Astyanax mexicanus*, a teleost with ancestral surface morphs (surface fish, SF) and derived cave morphs (cavefish, CF). We exposed SF to completely dark conditions and identified numerous altered traits at both the gene expression and phenotypic levels. Remarkably, most of these alterations mimicked CF phenotypes. Our results indicate that cave-related traits can appear within a single generation by phenotypic plasticity. In the next generation, plasticity can be further refined. The initial plastic responses are random in adaptive outcome but may determine the subsequent course of evolution. Our study suggests that phenotypic plasticity contributes to the rapid evolution of cave-related traits in *A. mexicanus*.

INTRODUCTION

A major problem in modern biology is understanding how organisms adapt to an environmental change and how complex, adaptive phenotypes originate. Phenotypic evolution can result from standing genetic variation, new mutations, or phenotypic plasticity followed by genetic assimilation, but these processes are often difficult to distinguish in slowly changing environments. This difficulty can be overcome by studying adaptation to more abrupt environmental changes, such as the dramatic transition from life on the Earth's surface to subterranean voids and caves.

A unifying feature of subterranean environments is complete darkness (1,2). Cave-adapted animals have evolved a range of unusual and specialized traits, often called troglomorphic traits, which enable survival in challenging conditions of the subsurface. In cave dwelling animals, visual senses and protection from the effects of sunlight are unnecessary, and consequently eyes and pigmentation are usually reduced or absent. To compensate for lack of vision, other traits, especially those related to chemo- and mechano-receptor sensations, are enhanced. Circadian rhythms that fine-tune organismal physiology with day-night cycles are also distorted, and light dependent behaviors, as well as the neural and endocrine circuits controlling these behaviors, are modified. Because photosynthetic organisms are not present in caves, primary productivity is absent and nutrient availability is usually limited. Survival under conditions of reduced and/or sporadic food resources is possible due to the evolution of modified feeding behaviors and adaptive changes in metabolism, such as lower metabolic rate, increased starvation resistance, and changes in carbohydrate and lipid metabolism (1).

The ancestors of cave dwelling animals originally lived on the surface. Regardless of whether the pioneering animals entered the subsurface accidently (by capture of surface waters or by falling into a pit) or purposely (to take refuge from harsh competition or changing climate conditions), they were suddenly exposed to darkness and subterranean living conditions. How did they overcome the challenges of life in darkness, such as orientation in the absence of light, survival during long periods without food, and detection of mates, in order to successfully establish underground lineages? The preadaptation hypothesis suggests that the ancestors of cave adapted animals were pre-adapted to darkness by already leading semi-nocturnal lifestyles in environments with low-light penetration, such as rotting logs, leaf litter, or in the soil, simplifying their transition to life in caves (3). However, many cave dwelling animals, such as the cavefish *Astyanax mexicanus,* are descended from diurnal surface-dwelling animals with no obvious pre-adaptations to low light. For these animals, the transition to dark subterranean habitats posed significant challenges.

In the present investigation, we used the teleost *Astyanax mexicanus* as a model system to understand the molecular, physiological and morphological basis of cave colonization. *A. mexicanus* is an excellent system for studying adaptation to cave life because it consists of both the ancestral-proxy form and multiple derived forms: surface dwelling (surface fish, SF)

and cave-dwelling conspecifics (cavefish, CF) respectively. Surface fish live in rivers exposed to day-night cycles with abundant food availability. In the karstic Sierra Madre Oriental del Norte mountain range in Mexico, SF have colonized about 30 caves (4), and their cave dwelling descendants have adapted to permanently dark, mostly food restricted subterranean waters.

Recent studies revealed that cavefish lineages are much younger than previously thought, from less than 20,000 (5) to about 200,000 (6) years old, implying that the evolution of numerous cave-related phenotypes may have occurred over relatively short time periods. Furthermore, troglomorphic phenotypes are maintained in *A. mexicanus* cavefish despite the lack of complete isolation between surface fish and cavefish populations (6–9). A remarkably recent origin of troglomorphic traits, also involving likely immigration from surface populations, has also been proposed for the evolution of cave-related traits in other cave dwelling animals, including invertebrates and vertebrates (10–15). What are the mechanisms that enable the rapid evolution of various distinct cave-related phenotypes, and how are they preserved despite the homogenizing process of gene flow from surface populations? Clearly, strong selection must be involved in these cases, but in order for selection to act, it is essential to have phenotypic variants with differences in fitness in the cave environment.

The conspecific surface and cave morphs of *A. mexicanus* are capable of hybridization in the laboratory and thus have been the subjects of extensive genetic analysis (16–18). However, not many mutations have been uncovered in CF, even in the presumably dispensable genes associated with eye degeneration, such as crystallin genes (19,20). A more immediate mechanism for survival is likely necessary when new colonizers first enter caves. This suggests the presence of standing genetic variation and/or phenotypic plasticity followed by genetic assimilation (21), rather than the complete reliance on the accumulation of new mutations to produce the necessary variability. An instance of standing genetic variation has been reported in *A. mexicanus* CF with respect to the evolution of eye reduction (22), but phenotypic plasticity has not been studied extensively in this system (but see (23)). This is surprising since phenotypic plasticity plays an important role in the adaptation of many different organisms to changing environments and the colonization of new habitats (reviewed in (24,25).

To replicate the colonization of the subterranean environment, we placed *A. mexicanus* SF in completely dark conditions for up to two years. By assaying different traits, we discovered that dark-raised SF show numerous phenotypes that are normally associated with CF adaptations to dark environments. The results imply that some cave-adapted traits may have appeared rapidly by phenotypic plasticity in *A. mexicanus*. Our results provide a basis for the evolution of adaptations in the cavefish lineage by genetic assimilation.

MATERIALS AND METHODS

Animals and Experimental Conditions

The study groups included *Astyanax mexicanus* SF and CF primarily from Pachón Cave (PA). In some experiments, CF from Tinaja (TI) and Molino (MO) caves were also used. Animals were obtained from the Jeffery Laboratory colony at the University of Maryland, and had undergone 3-5 generations in captivity since their original collection from the caves in 2002 (PA, TI) or 2006 (MO). Fish in the main colony were kept in 40 L tanks in cohorts of 8-15 animals and exposed to a 14-hour light/10-hour dark photoperiod. Spawning was induced every two weeks by extra feeding a few days prior to increasing the water temperature (26). Temperature regime changes were as follows: fish were normally kept in 22˚C, on the first day of spawning temperature was raised to 24˚C, on the second day raised to 26˚C, on the third day returned to 24˚C, and on the fourth day returned to 22˚C. Embryos were spawned in the night and collected in the morning by washing them from the breeding nets. Dead embryos were removed and the remainder transferred to clean fish system water containing methylene blue. The fish were raised and handled according to established University of Maryland and NIH guidelines and all experiments conform to the regulatory standards.

Fish embryos spawned at the same time from different parent tanks were mixed in order to minimize the effects of specific genetic backgrounds and mimic the situation in nature where fish are randomly swept into caves. Mixed embryo clutches were divided in half and randomly assigned to different experimental conditions. One group was initially exposed to darkness either as embryos or young fry (from 1-2 hours to 3 days after spawning) and then raised in complete darkness (D/D) for up to two years. The other group, originating from the same brood, was reared in a normal 14-hour light/10-hour dark photoperiod (L/D). All other conditions were identical between the D/D and L/D study groups. Fish were reared in plastic tanks with no filtration or running water. Depending on their size and age, fish were reared in 1, 3 or 7 liter tanks and transferred to larger tanks as they increased in size. Water was changed periodically at the same time for every tank in the experiment. Fish in each tank were fed the same volume of brine shrimp once per day. Dim red light (25 watts) was used when it was necessary to feed or handle the fish in the dark. Fish groups of the appropriate age were used on a per experiment basis. Fish used in a single experiment were collected for analysis at approximately the same time on the same day. Dead fish were removed when they were observed.

For the F1 generation experiments, SF placed in D/D as embryos and raised for 2 years were spawned using standard procedures as explained above. The spawned embryos (dSF) were divided in two groups within the first 24 hours post-fertilization, and one group was left in D/D conditions and the other group was placed in L/D photoperiod within first 24 hpf. A control group of SF spawned from L/D raised parents was also divided into two groups and raised in L/D or D/D during the period of experimentation.

116 Information on sample sizes, time lapsed in experimental conditions, and age of the fish
117 used in specific experiments is provided in the sections corresponding the description of
118 specific experimental methods.

119 The following procedure was used for collecting, and when necessary pulverizing, adult fish
120 for the respective experiments: fish were sacrificed with 0.4 or 0.5 mg/L MS222, tricaine
121 methanesulfonate (Western Chemical Inc., Ferndale, WA, USA). Fish were maintained in the
122 dark until they expired and then photographed, weighed, and pulverized in liquid nitrogen
123 using a mortar and pestle. The pulverized material was divided into several Eppendorf tubes
124 and stored at -80˚C until further processing. For the brain chemistry experiment, whole
125 brains were rapidly removed, placed in 1.5 mL Eppendorf tubes, immediately frozen on dry
126 ice and stored at -80˚C until analysis.

127 *Morphological Analysis*

128 After photography, the body length (total: rostrum to tail tip, fork: rostrum to tail fork, and
129 standard: rostrum to beginning of the tail), the dorsal-central width of the body (at the level
130 of the operculum and at the beginning of the dorsal fin), the eye diameter, and the pupil
131 diameter of 8 fish from each D/D and L/D experimental condition were measured using
132 ImageJ software.

133 Five fish from each group, kept under the experimental conditions described above for 9
134 months, beginning 2 hours post fertilization (hpf) were used for melanophore quantification.
135 Fish were fixed for 1 hr in 4% paraformaldehyde, washed three times in PBS, and examined
136 under a stereomicroscope. Pigment cells were counted in four different places on the left
137 side of each fish: in the proximal tail fin stripe, below the dorsal fin, along the dorsal flank of
138 the trunk, and on the chin. Eyes of four SF kept in D/D and three L/D controls kept in
139 experimental conditions for 1.5-2.5 years were embedded in paraplast blocks, sectioned,
140 stained, and morphometric analyses was performed on retinal layers (following (17)).

141 *RNA Sequencing, Differential Expression Analysis, and Identification of Enriched Pathways*

142 Three surface fish kept in D/D and three in L/D experimental conditions for 7 months post-
143 fertilization were used for RNA sequencing. Prior to RNA extraction, the stomach and liver
144 were removed from the fish because they compromised RNA quality. Total RNA was isolated
145 from samples stored in TRIzol reagent according to the manufacturer's instructions
146 (Invitrogen, Carlsbad, CA, USA). The fragment size, concentration, RNA integrity number
147 (RIN) and 28S/18S ratio of RNA extracts were determined using an Agilent 2100 Bioanalyzer
148 (Agilent, Santa Clara, CA, USA). The cDNA libraries were constructed using TruSeq mRNA
149 Library Prep Kit (Illumina, San Diego, CA, USA). Fragment sizes and concentrations of libraries
150 were verified using an Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR
151 machine (Thermo Fisher Scientific, , Waltham, MA, USA). The samples were sequenced in a
152 single Hiseq X-ten lane with a strategy of 150 bp paired-ends (PE), resulting in about 8 Gb
153 raw data for each sample. To avoid potential adapter contamination, the last 60 bp of all

154 reads were trimmed. Subsequently, the reads were removed if they contained: 1) more than
155 50% low quality (Q<35) bases; 2) more than 10 Ns, and 3) were PCR duplications. Cleaned
156 read quality was confirmed in FASTQC (27), and a total of 230,437,086 cleaned reads were
157 retained for transcriptomic analysis. The reads have been submitted to NCBI SRA (accession
158 numbers provided upon acceptance).

159 A local database was built on the Carbonate Cluster implemented by the National Center for
160 Genome Analysis Support (28) using the *Astyanax mexicanus* draft genome v102.93
161 (GCA_000372685.1) and associated gene model annotation files. The index of the reference
162 genome was built using Bowtie2 v2.3.2 (29), and paired-end clean reads were aligned to the
163 reference genome using TopHat v2.1.1, with max-intron-length set to 1000 (30). Transcript
164 coverage and differential expression were determined following the Cufflinks pipeline (31).
165 Briefly, mapped reads for each sample were assembled using Cufflinks v2.1.1 with the
166 parameters --frag-bias-correct, --multi-read-correct, --upper-quartile-norm, and --
167 compatible-hits-norm. Transcripts were merged using default Cuffmerge parameters, and
168 Cuffquant with --frag-bias-correct and --multi-read-correct was implemented to quantify
169 transcript expression. Significant changes between transcripts were calculated using Cuffdiff
170 (32) with parameters --compatible-hits-norm, --frag-bias-correct, and --multi-read-correct
171 for light and dark treatments. Results were visualized using the package CummeRbund (33)
172 implemented in R.Studio v1.0.136 (34) using R v3.5.1 (35).

173 Significantly differentially expressed genes (p_{adj}<0.1) were subjected to Gene Ontology (GO)
174 and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis as
175 implemented on DAVID Bioinformatics Resources (36,37) and pathways were investigated
176 more closely using Reactome (38).

177 *Real Time Quantitative RT-PCR*

178 Total RNA was isolated from 10 to 12-month old adult SF and PA placed in D/D or L/D
179 conditions within the first few hours after fertilization. After the liver and stomach were
180 removed, the RNA from the remaining body was isolated with Trizol Reagent (Thermo Fisher
181 Scientific, Waltham, MA, USA) according to the manufacturer's instructions and treated with
182 DNAse to remove genomic DNA if present. Equal amounts of RNA (1.5 µg) were reverse
183 transcribed using Superscript III or IV (Invitrogen, NY, USA). The cDNA concentration was
184 adjusted to 12.5 ng/µL and up to 50 ng were used in each reaction. We tested 12 different
185 HKG (housekeeping genes): (*ube2a*, *rpl13a*, *eef1a1l1*, *nek7*, *tbcb*, *rnf7*, *rpl27*, *ndufa6*, *ap5s1*,
186 *mob4*, *lsm12a*, *actc1b*) using RefFinder, which is located on the Cotton EST Database
187 webpage (39), and integrates currently available major computational programs (geNorm,
188 Normfinder, BestKeeper, and the comparative ΔΔCt method) to compare and rank the
189 candidate reference genes. The geometric mean of the 3 most stable HKG (*tbcb*, *mob4* and
190 *rnf7*) was used for normalization. Primers for genes of interest were designed across
191 exon/intron boundaries except for *pdx1*, where this was not possible (Supplementary Table
192 1). Primer efficiencies were calculated in Excel from standard curves, and those with

efficiencies between 90 and 110% were used for quantifications. The resulting PCR products were sequenced to confirm the targeted genes. Relative expression was calculated according to a formula $\Delta Ct = E \hat{}^{(Ct\ reference - Ct\ target)}$ (E = 1+primer efficiency/100) which enables the comparison of normalized expression of a gene between multiple samples, instead of ratios between two samples, taking primer efficiency into account. Statistical significance of observed expression differences was calculated by ANOVA and adjusted by Bonferroni index.

Starvation Resistance Determinations

SF and PA embryos were placed in L/D vs. D/D experimental conditions within 24 hpf. At 7 dpf (days post fertilization), the first day larval fish were fed brine shrimp, a group of 36 larvae from L/D and 36 from D/D per fish type was fed. Groups of 36 larvae from each experimental condition and each fish type were not fed. The fish were identically treated in all other aspects, and the number of living fish was recorded daily. Dead fish were removed each day.

Statistical analyses for survival analysis were carried out using R version 3.5.3 (R Core Team, 2019). Differences in starvation resistance were compared with a Cox proportional hazards model using the function coxph() from the R package 'survival' (40). For the parental generation, the model included the covariates fish (PA vs. SF), light status (D/D vs. L/D), and fish x light status. For the F1 generation, the model included the covariates fish (dSF vs. SF), light status (D/D vs. L/D) and fish x light status. If the null hypothesis that all $\beta = 0$ was rejected, *post-hoc* pairwise comparisons of contrasts were carried out using the function glht() from the package multcomp with the specification mcp = "Tukey" (41). Proportionality assumptions for Cox proportional hazards models were tested using the coxphz() function (40).

Metabolic Rate Determinations

Unhatched SF and PA embryos or 7 dpf larvae were placed individually in air-tight glass vials filled to the top with fish system water that contained no air bubbles. Half of the vials from each type were kept in L/D and the other half were wrapped in aluminum foil and kept in the same way in D/D. After two days, the O_2 remaining in each vial was measured using a Membrane Inlet Mass Spectrometer (MIMS) Machine (Bay Instruments, Easton, MD, USA). The amount of O_2 consumed per fish was calculated by subtracting the measured amount of O_2 in the vials with fish from the mean amount of O_2 measured in blank vials without fish. This experiment was repeated twice with embryos and once with larvae. The same methods were used in all replications. For embryos, N = 18 (SF L/D), 19 (SF D/D), 18 (PA L/D), 25 (PA D/D), and for larvae, N = 20 (SF L/D), 24 (SF D/D), 20 (PA L/D) and 24 (PA D/D).

Cortisol Quantification

We quantified cortisol from adult SF (N = 4) and PA (N = 4) that were maintained in D/D or L/D experimental conditions for 1.5 to 2 years beginning before 3 dpf. Cortisol was extracted

230 using previously developed procedures (42,43). Briefly, tissue samples stored at -80˚C were
231 thawed and homogenized in PBS after which 5 mL of diethyl ether was added. Following
232 centrifugation for 5 min at 3500 x g, the cortisol-containing top layer was removed and
233 evaporated in a fume hood overnight. The cortisol was reconstituted in PBS overnight at 4˚C
234 and quantified using the Cortisol ELISA Kit (Item № 500360, Cayman Chemical, Ann Arbor
235 MI, USA) following the kit protocol. Cortisol samples from fish within each group were
236 pooled and assayed in triplicate. The recorded absorbance was compared to a standard
237 curve to determine the cortisol concentration, and each sample was standardized to protein
238 concentration determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific,
239 Waltham, MA, USA).

240 *Hormone Quantifications*

241 Levels of growth hormone (GH) (CSB-E12121Fh) and thyroid stimulating hormone (TSH)
242 (CSB-EQ02726Fl) were quantified by ELISA using commercially available kits from Cusabio
243 (Wuhan, China). Adult SF kept in D/D for 1.5 years and PA kept in the experiment for almost
244 2 years were used. Three fish were used in each group, except for SF D/D in which 4 fish
245 were used. For comparison of hormone levels between different populations, 3 to 4-month
246 old SF (N=8), PA (N=5), TI (N=3), and MO (N=4) were used. The fish tissue was weighed, and
247 3 µl PBS/mg tissue was added to each sample. After two freeze-thaw cycles and
248 homogenization, the homogenate was centrifuged at 5000 x g for 5 min at 4˚C. Equal
249 volumes of supernatant from each fish type were pooled and immediately loaded on the
250 plate, 50 µl per well, in triplicate. Assays were performed following the manufacturer's
251 instructions and concentrations were determined according to the standard curve. Results
252 were standardized to protein concentration as described above.

253 *Triglyceride Quantifications*

254 We used three fish from each population (SF, PA) and treatment (D/D, L/D) aged
255 approximately 1 year. Pulverized fish were weighed and 20 µl per mg of 6% NP-40 (Abcam,
256 Cambridge, UK) was added to each sample. Samples were heated to 80-100°C for 2-5
257 minutes, and the process was repeated 2-3 times. Insoluble material was removed by
258 centrifugation for 2 minutes at 13,000 rpm, and equal volumes of supernatants from all
259 samples of the same fish type were pooled and diluted 20x in ddH_2O before ELISA. For ELISA,
260 50 µL of each sample was assayed in triplicate using the Triglyceride Quantification Assay Kit
261 (ab65336), (Abcam, Cambridge, UK) according to the manufacturer's instructions.
262 Concentrations were determined from the standard curve and standardized to protein
263 concentration as described above.

264 *Serotonin Quantification*

265 Brains were dissected from PA and SF kept in L/D or D/D experimental conditions for 1.5 to 2
266 years beginning at or before 3 dpf, and from adult SF (N=8), PA (N=5), TI (N=5), and MO
267 (N=6) kept in the main fish system and HPLC was used to quantify serotonin levels. In

268 addition, we quantified serotonin (5-HT) in samples of pooled 5 larvae (7 dpf) placed in the
269 L/D or D/D experimental conditions within first few hours after fertilization, N=6 (SF L/D), 5
270 (SF D/D), 5 (PA L/D), and 4 (PA D/D). Serotonin and its metabolite, 5-hydroxyindoleacetic
271 acid (5-HIAA), were analyzed using HPLC with electrochemical detection as described
272 previously with minor modifications (44). Brains were placed into 100 µL of sodium acetate
273 buffer (pH 5.0) containing the internal standard alpha-methyl dopamine (αMDA; Merck &
274 Co., Inc., Kenilworth, NJ). Fish bodies were placed in either 200 to 400 µL of acetate buffer
275 containing αMDA based on the amount of tissue present. Both tissue types were disrupted
276 by sonication using a 4710 Ultrasonic Homogenizer (Cole-Parmer Instrument Co., Chicago IL)
277 and stored at -80°C. Prior to analysis, the sonicated brain samples were thawed, 4 µL of 1
278 mg/mL ascorbate oxidase (Sigma-Aldrich, St. Louis, MO, USA) was added to each sample and
279 the samples were centrifuged at 17,000 g for 15 min. Fish body samples were treated the
280 same way except that the supernatant was centrifuged a second time through a 0.2 µm
281 filter. The filtered supernatant was removed and a Waters Alliance e2695 separation module
282 was used to inject 50 µL of the supernatant onto a C_{18} 4 µm NOVA-PAK radial compression
283 column (Waters Associates, Inc. Milford, MA) held at 30°C. The initial mobile phase (pH 4.1)
284 was prepared using 8.6 g sodium acetate, 250 mg EDTA, 14 g citric acid, 80 mg octylsulfonic
285 acid, and 80 mL methanol in 1 L of distilled water (monoamine standards and chemicals
286 were purchased through Sigma-Aldrich) and adjusted with small additions of octylsulfonic
287 acid, glacial acetic acid, and methanol to optimize the separation. Electrochemical detection
288 was accomplished using an LC 4 potentiostat and glassy carbon electrode (Bioanalytical
289 Systems, West Lafayette, IN, USA) set at a sensitivity of 0.5 nA/V (brain samples) or 1 nA/V
290 (body samples) with an applied potential of +0.7 V versus an Ag/AgCl reference electrode.
291 The pellet was solubilized in 400 µL of 0.4 N NaOH and protein content was analyzed using
292 the Bradford method (45). A CSW32 data program (DataApex Ltd., Czech Republic) was used
293 to determine 5-HT and 5-HIAA concentrations in the internal standard mode using peak
294 heights calculated from standards. Injection versus preparation volumes were corrected and
295 amine concentrations were normalized by dividing pg amine by µg protein. Data were tested
296 for differences using a Three Way Analysis of Variance (SigmaStat version 3.5, Systat
297 Software Inc., San Jose, CA). In analyses that revealed a significant effect between groups,
298 the Holm-Sidak method was used to conduct pairwise comparisons. All data were tested for
299 the presence of outliers using the Grubb's test (46). Based on this analysis two brain (PA D/D
300 night, SF L/D day) and three body samples (PA D/D night, PA L/D day, SF D/D day) were
301 deleted from the data set. In the end a total number of fish used in the analysis for brains
302 was 9 (SF L/D day), 10 (SF D/D day), 10 (PA L/D day), 10 (PA D/D day), 7 (SF L/D night), 6 (SF
303 D/D night), 4 (PA L/D night), 4 (PA D/D night) and for the bodies 10 (SF L/D day), 9 (SF D/D
304 day), 8 (PA L/D day), 10 (PA D/D day), 7 (SF L/D night), 7 (SF D/D night), 4 (PA L/D night), 5
305 (PA D/D night).

306 *The F1 offspring of Dark-raised Surface Fish*

307 We measured metabolic rate and starvation resistance in the F1 offspring of dark raised
308 (dSF) and control SF using the methods described above. Statistical analysis was also done
309 using the methods described above. N=24 larvae per group in starvation resistance
310 experiments and N=10 (L/D SF), 13 (D/D SF), 12 (L/D dSF) and 15 (D/D dSF) in metabolic rate
311 experiments.

312

RESULTS

Morphological Changes in Dark-raised surface fish

In this study, we exposed SF to D/D from early developmental stages until up to 2 years after spawning, and compared them to L/D controls. As a first step we compared body shape, length and width, eye size, and pigmentation in SF raised under D/D and L/D conditions (Fig 1A). We found no consistent differences in body parameters or eye sizes between D/D and L/D SF (Fig 1B). However, when we compared thickness of retinal layers between the two groups, 5 out of 7 layers were significantly different: D/D fish showed a thinning of the two plexiform layers and a thickening of the two nuclear layers and the photoreceptor layer (Fig 1D, Supplementary Fig 1). Surprisingly, we also observed a significantly higher number of melanophores in the flank of the trunk and below the dorsal fin in D/D compared to L/D SF, although similar levels of pigmentation were noted in other regions of the body (Fig 1C).

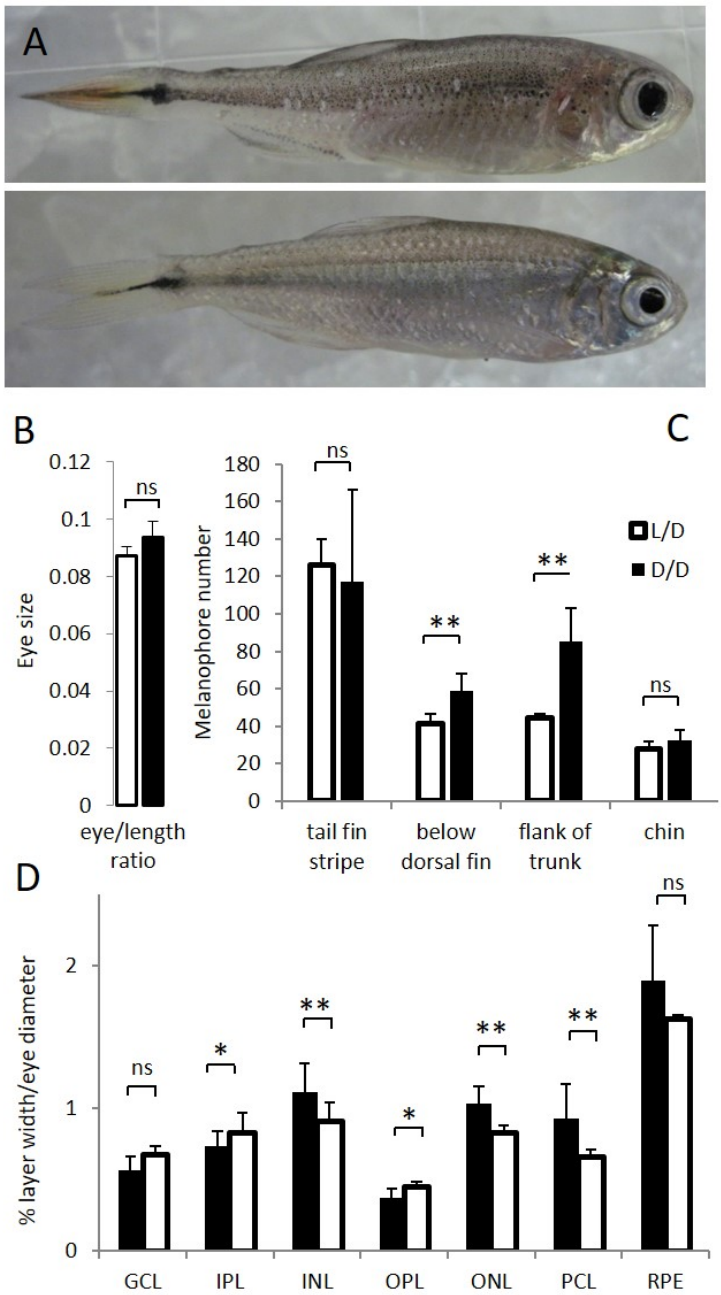
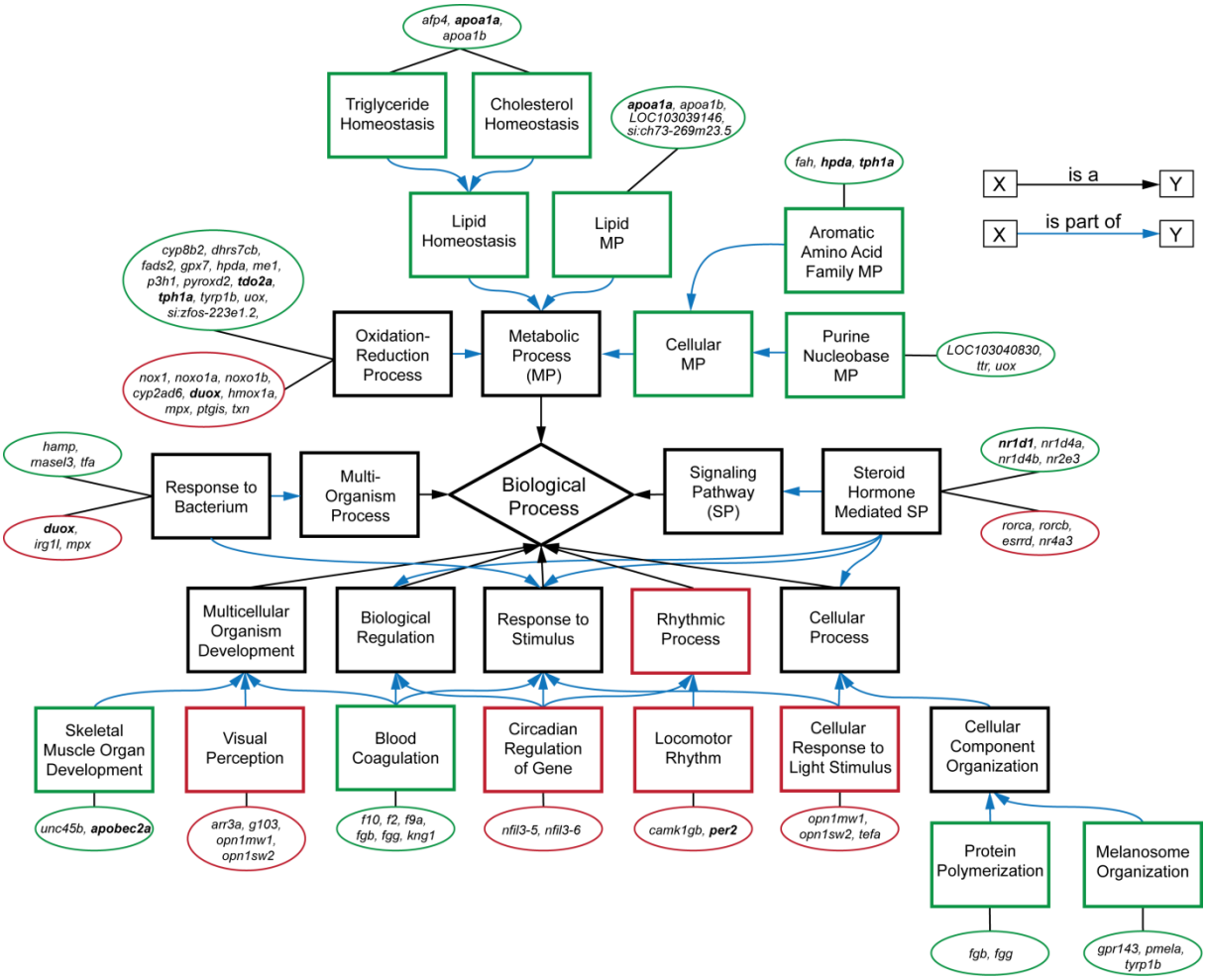


Figure 1. A. Surface fish (SF) kept in constant dark (D/D; top frame) vs. light/dark (L/D; bottom frame) photoperiod for 1 year. B. Eye size normalized by body length in D/D vs. L/D SF kept in the experimental conditions for 1 to 2 years. C. Number of melanophores in 1 year-old D/D vs. L/D SF determined in 4 different body regions. D. Thickness of retinal layers in D/D vs. L/D fish: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PCL photoreceptor cell layer; RPE, retinal pigment epithelium measured as a ratio to eye diameter. (Error bars: SD; T-test Ns – not significant, *p<0.05, ** P<0.001).

Gene Expression Changes in Dark-raised Surface Fish

To understand the effects of exposure to constant darkness at the molecular level, a comparative transcriptomic analysis was conducted. We used three, 7 month-old surface fish placed in either D/D or L/D conditions within 1 dpf. RNA sequencing yielded 21.9 to 27.6 million reads from each fish, representing a total of 25,194 genes. Basic statistical data are included in Supplementary Table 2. We found 356 differentially expressed genes (DEG) at a significance threshold of $p_{adj}<0.1$ (Supplementary Table 3). Of these, 210 were up-regulated and 146 were down-regulated in the D/D fish; the set of DEGs contained 67 genes with unconfirmed functions. We were particularly interested in the genes related to known aspects of the CF phenotype. Genes involved in circadian regulation, locomotor rhythm and visual perception were down-regulated in D/D fish, whereas lipid metabolism was the main functional category enriched in the up-regulated gene set. Two genes involved in pigmentation were also changed but their expression was higher in D/D, consistent with the increase in pigment cells described above. We also found significant changes in gene expression which were not predicted from known CF-associated phenotypes. These genes function in oxidation-reduction processes, hormone activity, hemostasis, aromatic amino acid metabolism, gene expression, metabolism of proteins, and signal transduction (Fig 2).



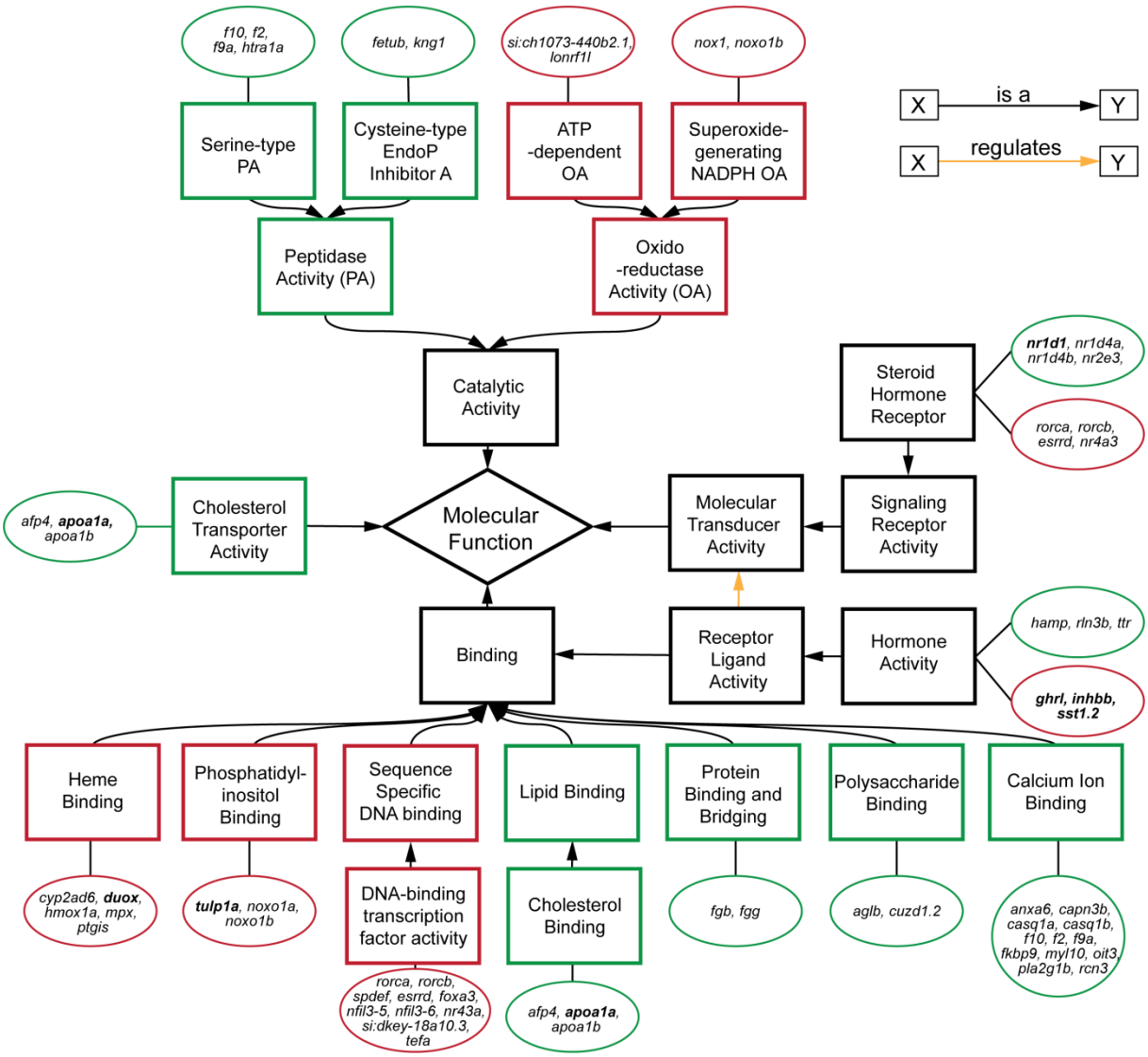
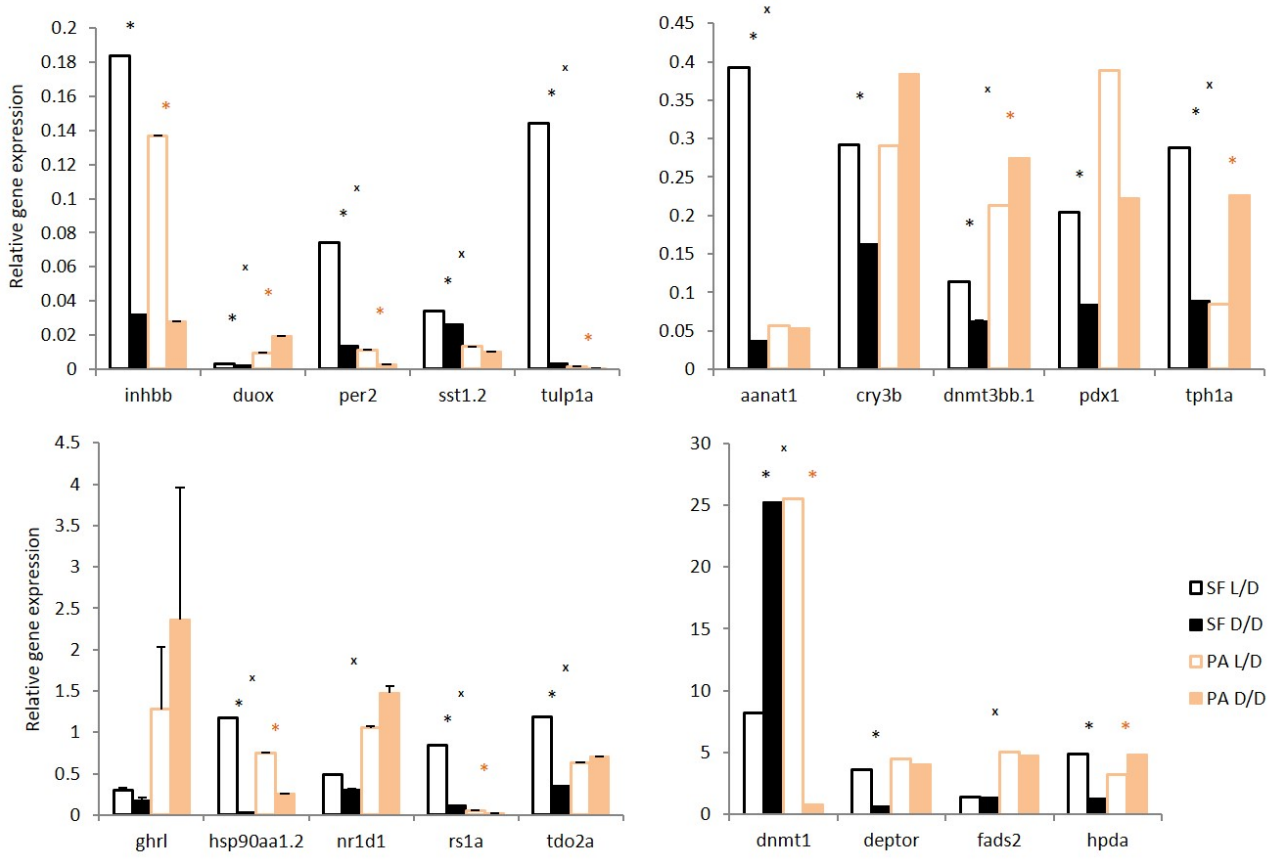


Figure 2. Subset of relevant, enriched GO terms (boxes) for biological processes (top) and molecular functions (bottom) of DEGs (circles) in the transcriptome. Genes tested by RT-PCR are in bold. Red outlines are down-regulated terms and genes, and green outlines are up-regulated.

To validate the RNAseq results, we examined the levels of several DEG by real time PCR (RT-PCR) (Fig 3). We confirmed the down-regulation of the hormone related genes *somatostatin 1 tandem duplicate 2* (*sst1.2*) and *inhibin beta B* (*inhbb*), ros involved *dual oxidase* (*duox*), the circadian rhythm genes *period circadian clock 2* (*per2*) and *cryptochrome-1-like* (*cry3b*), the vision related genes *retinoschisin 1a* (*rs1a*) and *tubby like protein 1a* (*tulp1a*), as well as genes involved in the regulation of metabolism *pancreatic and duodenal homeobox 1* (*pdx1*), and *deptor* in D/D compared to L/D SF. However, the tyrosine metabolism gene *hydroxyphenylpyruvate dioxygenase a* (*hpda*), and the tryptophan metabolism genes *tryptophan 2,3-dioxygenase* (*tdo2a*), *aralkylamine N-acetyltransferase 1* (*aanat1*), and *tryptophan hydroxylase 1a* (*tph1a*) showed down-regulation according to RT-PCR results,

371 whereas they were up-regulated in the transcriptome. This discrepancy may be related to
372 differences in the age or condition of fish at the time of sampling.

373 In addition, we quantified some genes which did not show significant differential expression
374 according to RNAseq results but are important for the cavefish phenotype: *heat shock*
375 *protein 90α* (*hsp90aa1.2*), and the *de novo* DNA methyltransferases *dnmt1*, *dnmt3aa*,
376 *dnmt3ab*, *dnmt3ba* and *dnmt3bb. Hsp90aa1.2, dnmt1* and *dnmt3bb* showed changes in
377 expression related to photoperiod in *Astyanax*. The RNAseq results and subsequent RT-PCR
378 validation showed changes in the expression of DEG relative to photoperiod in both SF and
379 PA CF. Expression of some genes changed in the same direction in both SF and CF (e.g. *inhbb*,
380 *per2*, *sst1.2*), whereas others changed in one and not the other fish type, or changed in the
381 opposite directions in different fish types (e.g. *duox*, *nr1d1*, *tph1a*). Except for *duox* and
382 *dnmt3bb.1*, all of the significantly changed genes show the same direction of changes in L/D
383 vs. D/D SF and in SF vs. PA: *hsp90aa1.2*, *rs1a*, *tdo2a*, *dnmt1*, *per2*, *sst1.2*, *tulp1a*, *aanat1*, and
384 *tph1a.*



385

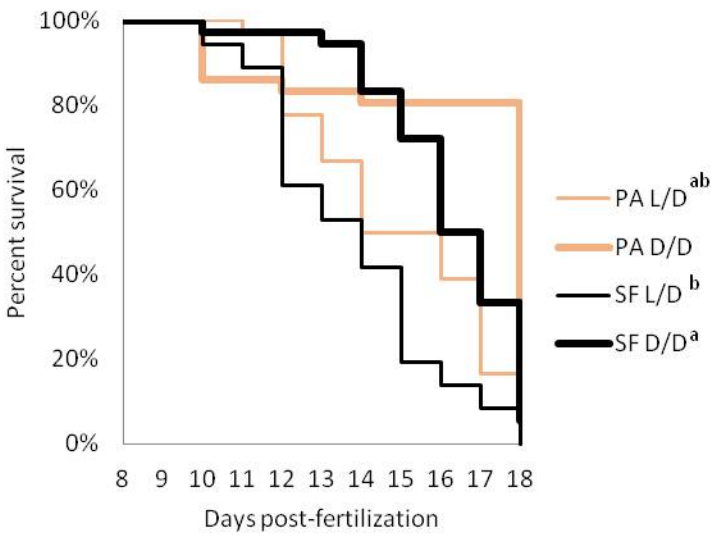
386 **Figure 3**. Normalized relative expression levels of genes in D/D and L/D SF and PA
387 determined by RT-PCR. (Error bars: SD; ANOVA with Bonferroni adjustments p<0.05 * for SF
388 D/D vs. SF L/D; * for PA D/D vs. PA L/D; ^{X} for SF vs. PA,).

389

390 ***Starvation Resistance in Dark-raised Surface Fish***

391 A major challenge facing animals that colonize caves is low food availability due to limited or
392 absent primary productivity. To determine how a surface ancestor may have coped with this
393 difficulty, we raised SF and PA larvae in D/D and L/D beginning <24 hours post fertilization
394 (hpf) without feeding (N=36 larvae/group). D/D and L/D controls were fed daily portions of
395 brine shrimp from 7 dpf, when larvae normally lose their reliance on yolk and begin feeding.
396 By 15 dpf, about 25% more SF and over 65% more PA unfed larvae were alive in D/D
397 compared to L/D conditions. By 18 dpf, only a few unfed embryos were still alive, and they
398 were all from the D/D conditions (8 PA and 2 SF) (Fig 4). The results show that dark raised SF
399 and CF are more resistant to starvation than siblings raised under a normal photoperiod and
400 that PA larvae survive starvation better than SF larvae regardless of the lighting conditions.

401



402

403 **Figure 4.** Survival curve of starvation resistance in *Astyanax mexicanus* surface fish (SF) and
404 Pachón cavefish (PA) raised in complete darkness (D/D) or a normal photoperiod (L/D).
405 Graphs show the percent of surviving fish (from the initial 36) on each day. Groups of SF and
406 PA larvae from each condition were starved starting at 7 dpf. Vertical drops represent
407 individuals lost at a given time point. Groups in the legend that share a superscript are not
408 statistically different, p values calculated by Cox proportional hazards model followed by
409 generalized linear hypothesis test.

410

411 ***Metabolic Rate Decrease in Dark-raised Surface Fish***

412 We hypothesized that fish in D/D survived starvation longer because of lower energy
413 expenditure. To test this possibility, we exposed SF and PA to D/D vs. L/D conditions within
414 first 24 hpf after spawning and measured O_2 consumption at 2.5 dpf and 7.5 dpf. The results
415 showed that SF and PA larvae raised in D/D have decreased metabolic rates when compared

to SF and PA larvae raised in L/D conditions. This result was found to be significant in SF each time this experiment was conducted (3 replicates) for both 2.5 and 7.5-day-old larvae. Furthermore, D/D PA showed a decrease in O_2 consumption at 2.5 but not at 7.5 dpf when compared to larvae raised in L/D. In addition, PA and SF metabolic rates were not significantly different at 2.5 dpf, whereas at 7.5 dpf PA has a lower metabolic rate than SF larvae raised in L/D conditions. At 2.5 dpf metabolic rate is similar between SF and PA, and darkness caused it to be reduced in both types of fish. However, by 7.5 dpf the metabolic rate in PA was reduced compared to L/D SF, and PA did not show a plastic response to darkness, whereas at the same age SF metabolic rate was still affected by darkness (Fig 5). In summary, the results support the hypothesis that D/D fish survive starvation longer because of lower metabolic rates.

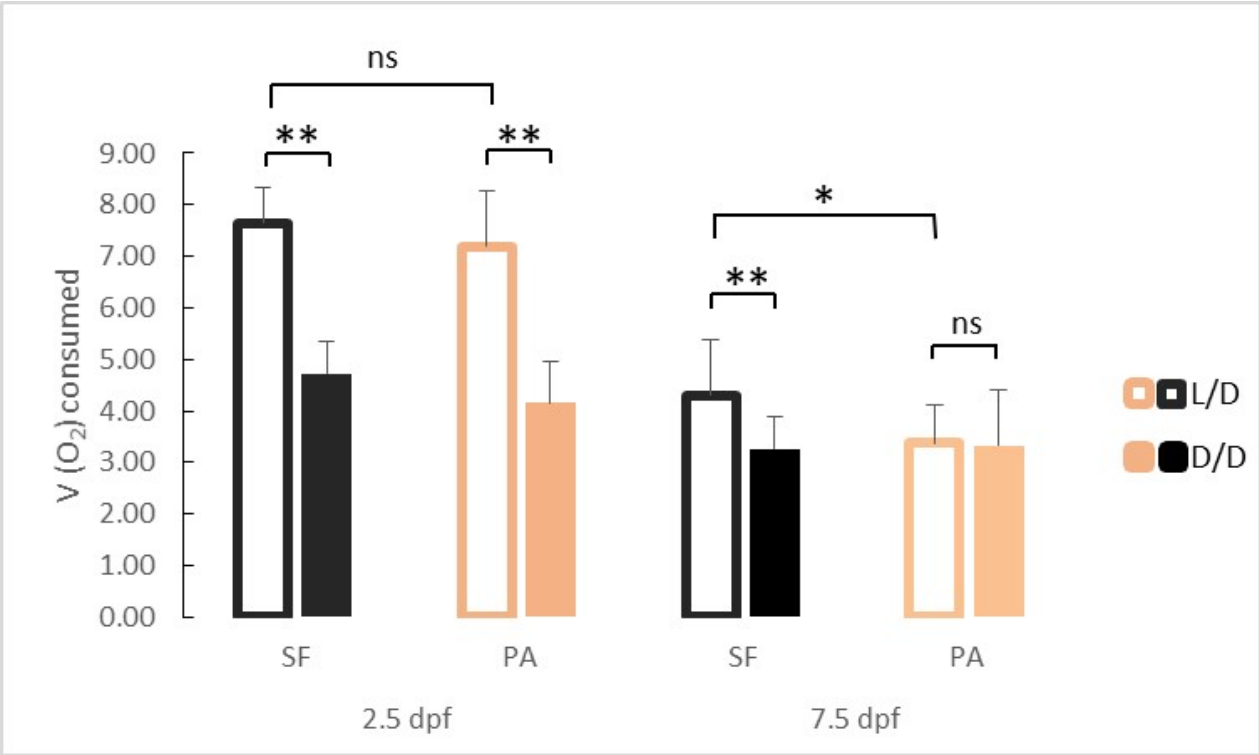
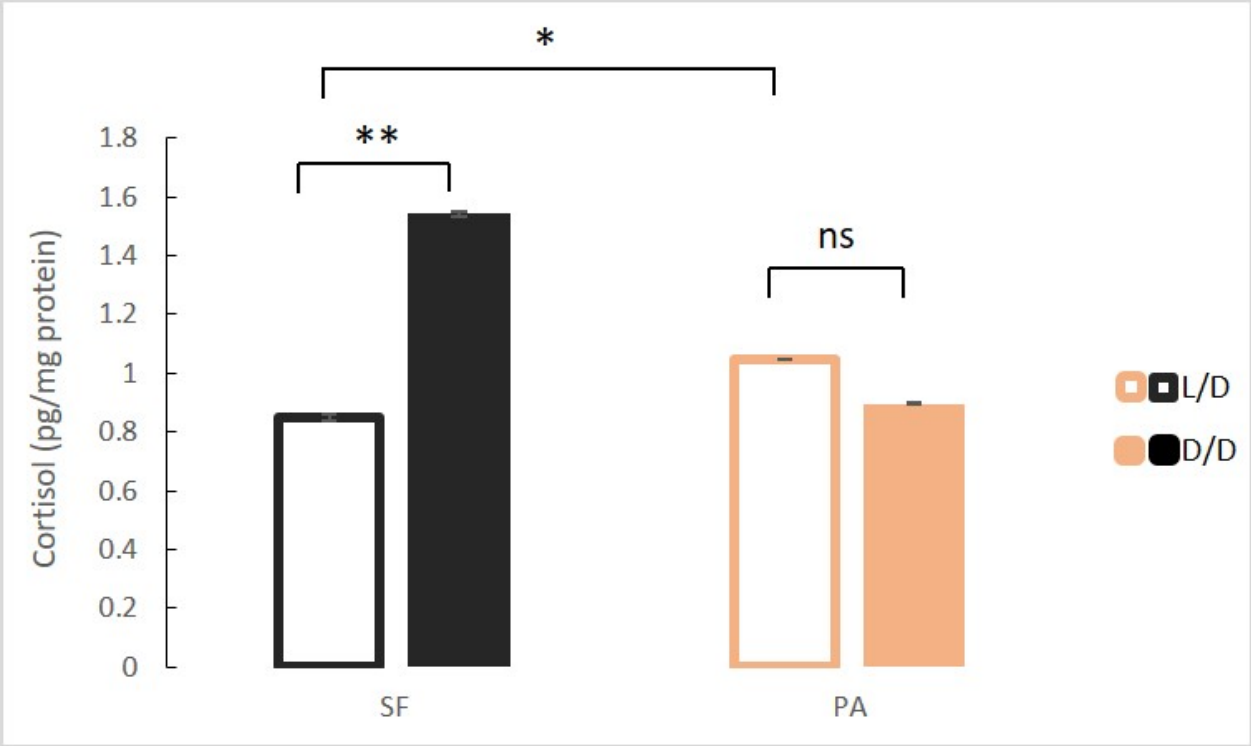


Figure 5. Average oxygen consumption at 2.5 and 7.5 dpf in SF and PA larvae kept in D/D versus L/D conditions (N = 18 - 25 larvae/group). (Error bars represent standard deviation, ns: not significant, *p<0.05, **p<0.01 as calculated by ANOVA and Tukey HSD Test.)

Cortisol Increases in Dark-raised Surface Fish

We hypothesized that exposure to constant darkness may represent a chronic stressor and tested this possibility by comparing cortisol levels in both L/D and D/D SF and PA. Surface fish raised in D/D conditions had significantly higher cortisol levels than L/D SF (Fig 6). In contrast, PA did not show a significant change in cortisol levels after exposure to D/D. This

438 result was confirmed in three biological replicates using fish of different ages and different
439 periods of dark exposure and shows that dark raised SF exhibit higher cortisol levels than SF
440 raised on a normal photoperiod.



441

442 **Figure 6.** Mean cortisol levels in adult surface fish (SF) and Pachón cavefish (PA) kept in D/D
443 or L/D conditions for 1.5 to 2 years (N=4/group). (Error bars represent SD in three technical
444 replicates, ANOVA and Tukey HSD Test: Ns – not significant, *p<0.05, **p<0.01).

445

446 ***Fat Increase in Dark-raised Surface Fish***

447 Our RNAseq results show that genes involved in many aspects of fat metabolism were up-
448 regulated in D/D versus L/D reared SF. To confirm this on a phenotypic level, we quantified
449 triglyceride content in SF and PA raised to adulthood under D/D and L/D conditions. In fish
450 raised under L/D conditions, PA had higher triglyceride levels than SF. Surface fish raised in
451 D/D conditions had markedly higher triglyceride levels than L/D SF. Triglyceride levels in D/D
452 PA were also higher than levels measured in L/D PA, although the difference is more modest
453 than that observed for SF (Fig 7). These results suggest that dark raised SF have higher levels
454 of triglyceride metabolism that SF raised on a normal photoperiod.

455

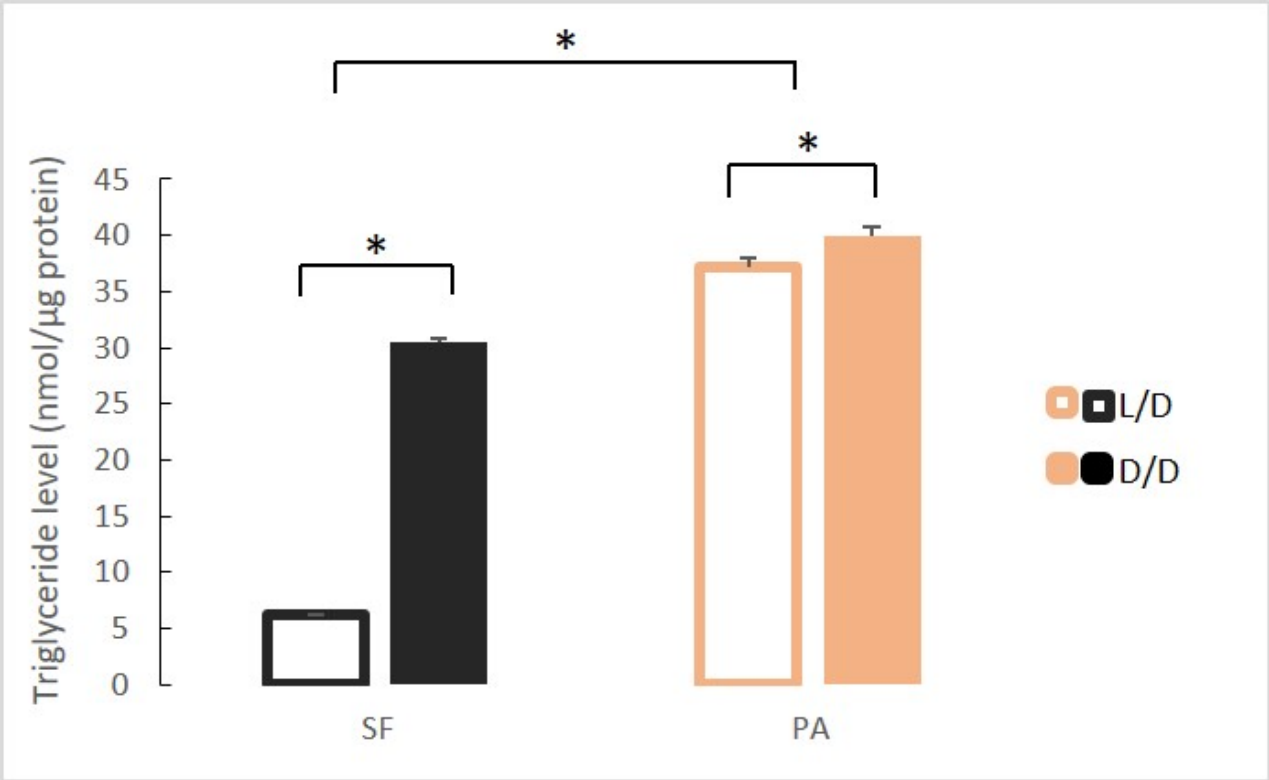


Figure 7. Mean triglyceride levels in SF and PA raised under D/D versus L/D conditions for approximately 1 year since < 24 hpf. (Error bars represent standard deviation. *p<0.01; ANOVA and Tukey HSD Test).

Hormone Levels Change in Dark-raised Surface Fish

Because of morphological changes in the pituitary and thyroid glands in dark raised SF (47) and differential expression of some of the genes associated with pituitary hormones (e.g. *sst1.2*, *inhbb*, *ghrl*) in the dark-raised SF transcriptome, we quantified the levels of pituitary hormones TSH and GH in SF and PA adults raised in either L/D or D/D conditions. TSH levels were lower in D/D SF ($p<0.05$) when compared to L/D SF. In contrast, TSH levels in PA raised in L/D and D/D conditions were similar, and PA had higher TSH levels than SF regardless of lighting conditions (Fig 8A). When TSH levels were compared between SF and three populations of CF, there was a trend for higher TSH levels in TI ($P=0.07$), but not MO ($P=0.16$), and TSH levels where significantly higher in PA (Fig 8B). The levels of GH were higher in both SF and PA raised in D/D conditions when compared to fish raised in L/D (Fig 9A). All three populations of CF had higher GH levels than SF (Fig 9B).

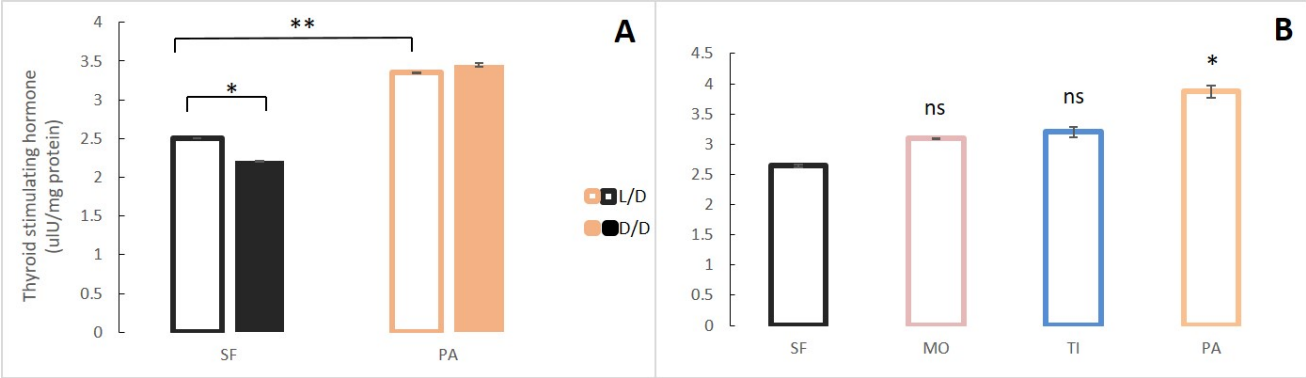


Figure 8. A. Mean Thyroid stimulating hormone (TSH) levels normalized by protein concentration in adult surface fish (SF) and Pachón cavefish (PA) kept in D/D or L/D conditions for 1.5 to 2 years. B. Mean TSH levels in SF and 3 different CF populations: Pachón (PA), Tinaja (TI) and Molino (MO) caves. (Error bars represent SD in three technical replicates. N ranges from 3-8 fish/group; * $p<0.05$; ** $p<0.01$ as calculated by ANOVA and Tukey HSD Test. In B. ns or * denotes significance in comparison to SF.)

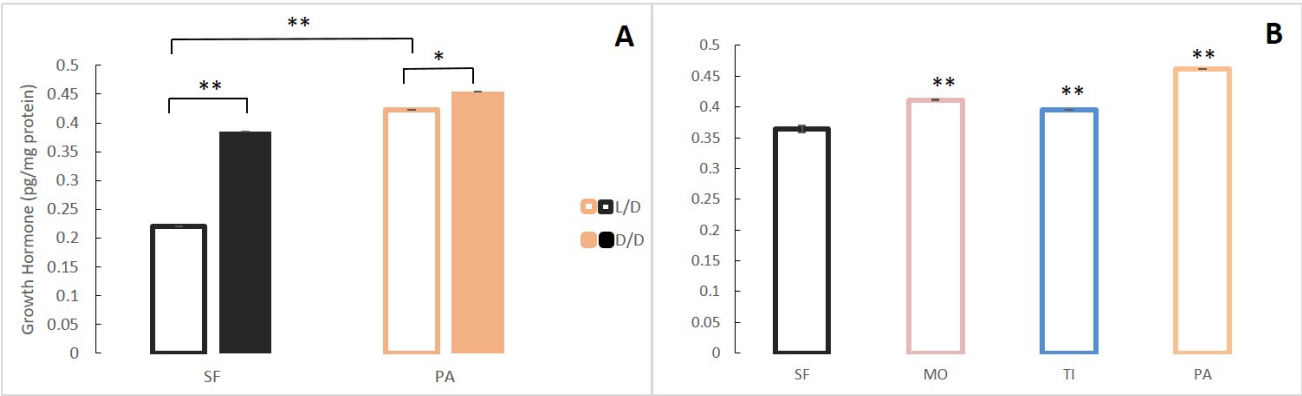


Figure 9. A. Mean growth hormone (GH) levels normalized by protein concentration in adult surface fish (SF) and Pachón (PA) cavefish kept in D/D or L/D conditions for 1.5 (SF) and 2 years (PA) since < 3 dpf. B. Mean GH levels in 3-4 month old SF and 3 different CF populations: PA, Tinaja (TI), and Molino (MO). (Error bars represent SD in three technical replicates. N = 3 to 8/group. *$p<0.05$; **$p<0.01$ as calculated by ANOVA and Tukey HSD Test. In B, **$p<0.01$ compared to SF).

Serotonin Decrease in Dark-raised Surface Fish

According to the RNAseq analysis, the tryptophan metabolism pathway was significantly up-regulated in D/D versus L/D SF, while real time qPCR suggested lower expression of the *tdo2a*, *aanat1* and *tph1a* genes in dark–raised SF. Therefore, we compared serotonin (5-HT) levels in D/D and L/D SF and PA adults by HPLC. Since serotonin levels fluctuate according to a daily rhythm (48), samples for this assay were collected between 11 AM and 3 PM (day) or 11 PM and 3 AM (night) regardless of rearing conditions. Serotonin concentrations were

lower in the brains (Fig 10A) and the bodies (Fig 10B) of D/D compared to L/D SF. Likewise, the 5-HT metabolite 5-HIAA was lower in the brain of D/D SF (Supplementary Fig 2). It was not possible to quantify 5-HIAA in the body because it was masked by several interfering peaks. Serotonin levels in the SF brain, but not the body, were significantly lower at night when compared to samples collected during the day. In PA brains collected during the day, 5-HT levels were lower in fish raised in D/D when compared to L/D conditions but 5-HT levels were similar in D/D and L/D brains at night. Modest, but significant, changes in body 5-HT were evident in PA with increases in D/D relative to L/D collected during the day, and the opposite change occurred in bodies collected during the night. Serotonin levels were significantly lower in PA brains and bodies when compared to SF regardless of experimental conditions (D/D, L/D, day/night). Comparison of SF brain 5-HT with TI, MO and PA fish showed that all three cave populations have lower brain 5-HT (Fig 10C). In contrast to adults, 5-HT was significantly higher in PA compared to SF at 7 dpf, and darkness did not affect 5-HT levels in either larval fish types (Fig 10D).

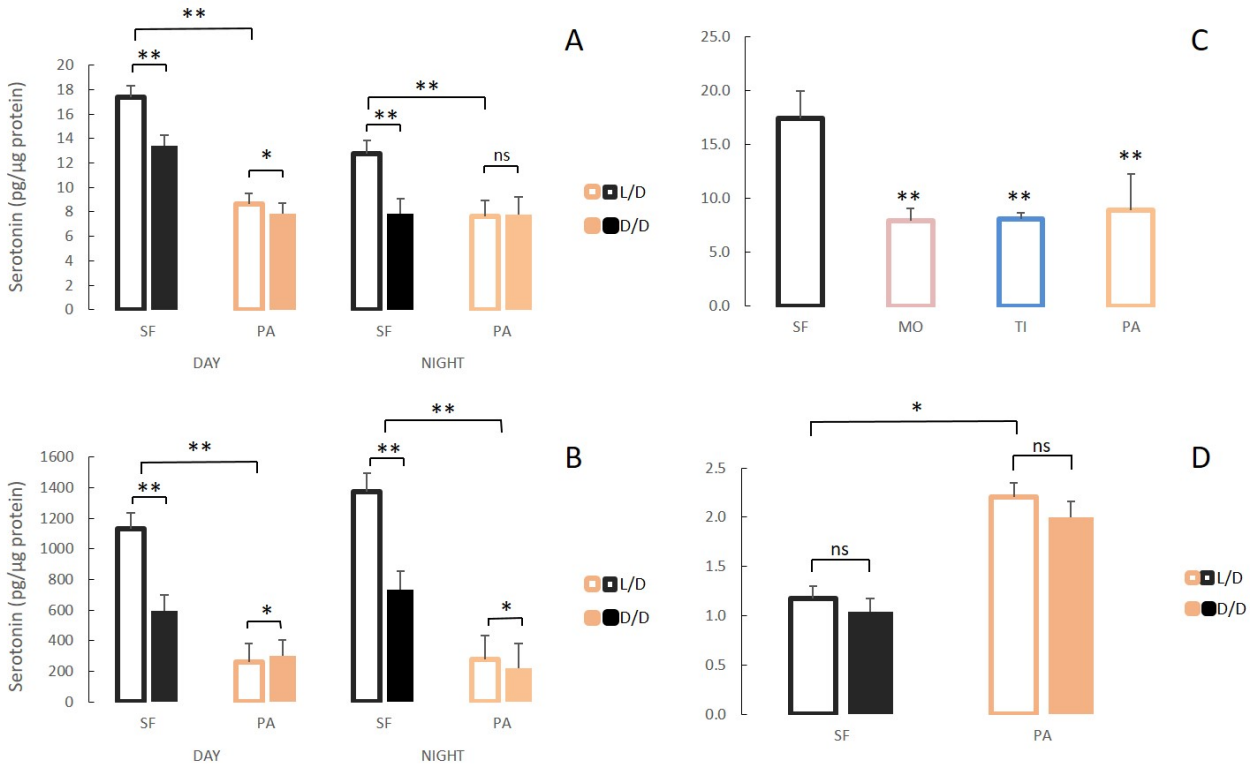


Figure 10. Serotonergic system changes in adults and larvae of light/dark (L/D)- and dark/dark (D/D)-reared surface fish and cavefish. A, B. Serotonin levels in adult brains (A) and bodies (B) of D/D and L/D reared surface fish (SF) and Pachón cavefish (PA) collected in the middle of the day (DAY) and the middle of the night (NIGHT). (Error bars represent the standard error of the means.) C. Mean serotonin levels in brains of adult SF and 3 different CF populations: Molino (MO), Tinaja (TI) and PA. D. Mean serotonin levels in pooled samples of 5 larvae aged 7 dpf placed in the experiment within first few hours post fertilization. (Error bars SEM; ns – not significant, *p<0.05; **p<0.01 as calculated by ANOVA and post-

hoc Tukey HSD Test. In C, **p<0.01 vs SF. The number of each fish type subjected to analysis ranged from 4 to 10 per group.)

Progeny of Dark Raised Surface Fish Show Plasticity in Starvation Resistance and Metabolic Rate

To test whether the plastic changes in the fish reared in the dark during the first generation are maintained and transferred to the next generation, we induced spawning in D/D reared SF. We measured starvation resistance and metabolic rate in F1 embryos (dSF) developing in D/D or in L/D during the second generation, as well as the offspring of L/D reared controls exposed to L/D or D/D conditions. The F1 larvae reared in L/D showed no differences in starvation resistance (Fig 11) or metabolic rate (Fig 12), regardless of whether they were the progeny of L/D or D/D parents. Their cohorts raised in darkness showed a modification in these phenotypes: dSF larvae derived from D/D parents showed slightly enhanced plasticity and a minor shift toward lower metabolic rate and higher starvation resistance (although not statistically significant) than larvae raised from L/D parents.

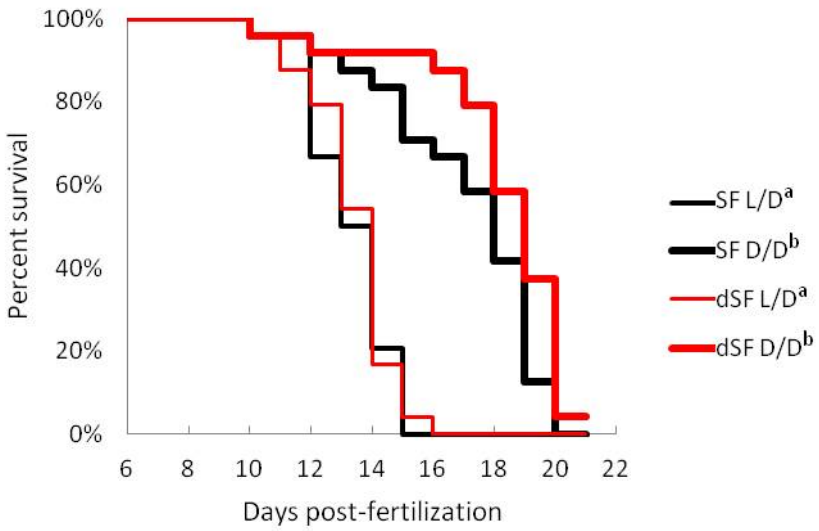
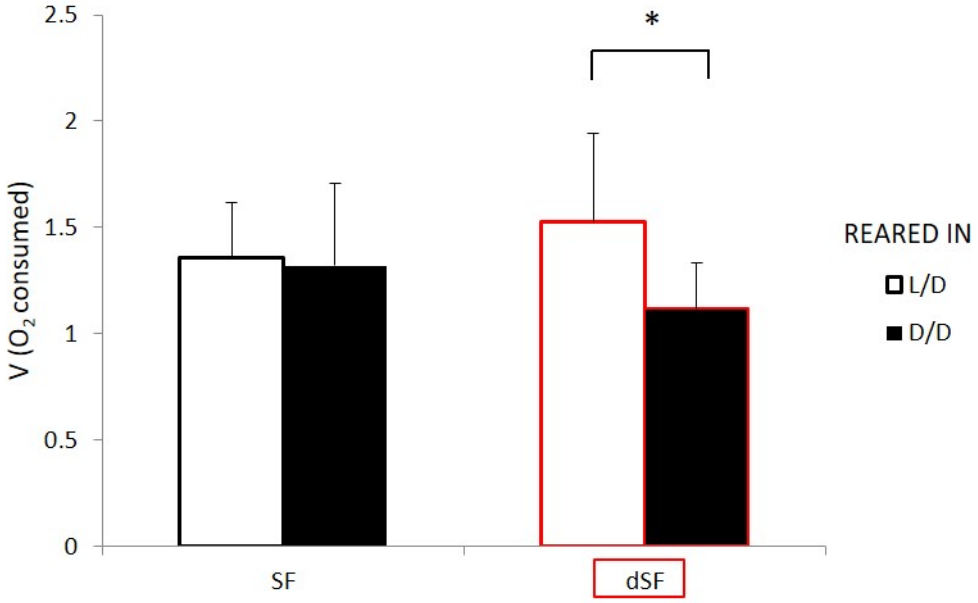


Figure 11. Survival curve of starvation resistance in F1 offspring of surface fish kept in normal light/dark photoperiod (SF) and surface fish raised in total darkness for 2 years (dSF). Graphs show the percent of surviving fish (from the initial 24) on each day. A. One group of larvae from each fish type (SF, dSF) and each lighting condition (D/D, L/D) was starved starting at 7 dpf (a vs. b p<0.0001). Vertical drops represent individuals lost at a given time point, groups in the legend that share a superscript are not statistically different, p values calculated by Cox proportional hazards model followed by generalized linear hypothesis test.



546

547 **Figure 12.** Average oxygen consumption of 11 dpf F1 offspring surface fish kept in the
548 normal light/dark photoperiod (SF) and surface fish kept in total darkness for 2 years (dSF).
549 Each group of offspring was exposed to D/D or L/D conditions within first 24 hpf. (Error bars
550 represent standard deviation; *$p<0.05$, as calculated by ANOVA and Tukey HSD Test.).

551

552 **DISCUSSION**

553 Contrary to the widely accepted view that cave-adaptations result from long-term genetic
554 processes (49–51), our results indicate that some cave-related traits can appear within a
555 single generation by phenotypic plasticity. Exposure to constant darkness can trigger rapid
556 metabolic, neurological, morphological and molecular changes necessary for survival of *A.*
557 *mexicanus* SF colonizers, which may have allowed for the adaptations evident in extant CF.
558 The initial plastic responses can be adaptive or non-adaptive. Subsequently, in the case of an
559 adaptive response, plasticity would be further selected for, or in the case of non-adaptive or
560 mal-adaptive responses, plasticity would be selected against. Our findings strongly implicate
561 phenotypic plasticity as an important mechanism of cave colonization and rapid evolution of
562 cave-related traits and open the possibility that genetic assimilation may be an underlying
563 mechanism of adaptive evolution in *A. mexicanus* cavefish.

564 Phenotypic plasticity is often the first response of organisms exposed to an environmental
565 change. The ability to quickly respond to novel environmental conditions is essential for
566 initial survival and, eventually, for adaptation to occur on the genetic level (24,25,52).
567 Darkness is a hallmark of the subterranean realm, the initial stress new cave colonizers
568 encounter, and the only environmental component shared by all underground habitats (2).
569 Building on the work of Rohner et al. (22), we hypothesized that stress caused by perpetual
570 darkness may be an important facilitator of the rapid appearance of novel and beneficial
571 traits that enabled ancestral SF to endure the environmental shift associated with their entry
572 and ultimately their adaptation to life in caves. To test this hypothesis, we used
573 contemporary *A. mexicanus* SF as a proxy of the ancestral SF colonizers, and exposed them
574 to complete darkness to simulate the most important change that the original SF ancestors
575 of CF experienced when colonizing caves. Indeed, we found an increase in cortisol level in
576 D/D exposed SF supporting our initial premise of darkness activating a stress response.

577 We expected to find plastic changes in D/D reared SF involving pituitary and thyroid
578 hormonal regulation, adipose tissue, and body shape based on the previous work on dark-
579 raised *A. mexicanus* SF, Chica CF, and Los Sabinos CF (47). Furthermore, experiments on
580 *Poecillia mexicana*, another teleost with cave adapted lineages, showed that darkness
581 caused degenerate changes in the spine and promoted sexual isolation between adjacent
582 surface and cave lineages (53–55). Although our study was unable to replicate the changes in
583 body shape seen by Rasquin (1949), we confirmed her observation of increased body fat. We
584 also confirmed changes in pituitary and thyroid hormone metabolism, both in gene
585 expression and hormone levels. Down-regulation of *sst1.2*, a homologue of somatostatin -
586 growth hormone-inhibiting hormone, is consistent with increased GH in D/D SF. In addition,
587 the changes in TSH and GH levels we observed in dark raised SF are consistent with the
588 reduction of basophils and the predominance of acidophils in the pituitary gland of D/D SF
589 reported previously (47). These results suggest that one of the immediate responses to cave
590 colonization and darkness may be changes in the endocrine system. In fact, endocrine

591 signaling, along with epigenetic changes, activation of heat shock proteins, and
592 transcriptional regulation, are the major molecular mechanisms involved in regulating plastic
593 responses (56–58). Our transcriptome analyses revealed that genes and GO terms involved
594 in all of the above processes showed significant changes in response to darkness. In addition
595 to hormonal signaling, there are changes in genes involved in the stress response, several
596 different signal transduction pathways, protein metabolism including protein folding and
597 posttranslational modifications, and genes showing transcription factor or epigenetic
598 regulator activities. Furthermore, using RT-PCR we found that the expression of *hsp90aa1.2*,
599 *dnmt1* and *dnmt3bb*, genes related to stress or epigenetic processes (59) were changed in
600 D/D SF. These results suggest that multiple molecular mechanisms underlying phenotypic
601 plasticity may be mobilized by exposure of SF to darkness.

602 Our RNAseq results contained functional categories enriched in both the up- and the down-
603 regulated dataset: hormone activity (as mentioned above) and oxidation-reduction
604 processes. Changes in oxidation-reduction processes may be related to increased stress
605 following the exposure to a changed environment. Alternatively, these changes may be light-
606 dependent since light activates the production of reactive oxygen species (ROS) which, in
607 turn, act as messengers between photoreception and the circadian clock (60,61). Pathways
608 involved in the response to oxidative stress, photoreception, and circadian regulation were
609 down-regulated. Thus, the absence of light may directly reduce ROS production,
610 photoreception, and circadian control by modifying a single signaling cascade.

611 Our study revealed many traits that change in SF upon exposure to darkness. Strikingly, all
612 these traits are associated with the cave lifestyle, and almost all of them change to resemble
613 CF adaptive phenotypes. For example, compared to SF, CF have higher starvation resistance
614 (62), lower metabolic rate (63,64), higher basal cortisol level (43), and lower serotonin levels
615 (but see 62). When exposed to darkness, in addition to mimicking higher GH and triglyceride
616 levels in CF (see above), we found that SF show higher starvation resistance, lower metabolic
617 rates, higher cortisol levels, and lower serotonin levels. The results demonstrate that for
618 these traits SF begin to resemble CF in the same generation following exposure to complete
619 darkness. Because the plastic phenotypes are under genetic control in multiple
620 independently evolved CF populations and irreversible when CF are transferred to light in
621 the laboratory (4,66), the results are consistent with the possibility that the initial dark-
622 induced plastic responses in SF became fixed in CF-like descendants via genetic assimilation.
623 However, not all CF traits could be replicated by exposing SF to darkness. Therefore,
624 although important, plasticity caused by constant darkness is not the only path for the
625 appearance of cavefish traits. Other mechanisms, including plasticity caused by
626 environmental factors other than darkness (e.g. changes in nutrient availability), standing
627 genetic variation, new mutations, or any combination of these processes, might also be
628 involved.

629 Although darkness is an ecologically relevant cue, the induction of changes in SF by the
630 absence of light does not necessarily imply that they are adaptive for cave life. Plasticity can
631 result in adaptive, non-adaptive as well as maladaptive responses (67,68). It is self-
632 explanatory how increased triglyceride content, starvation resistance or decreased
633 metabolic rate can increase fitness in the (food-limited) cave environment, but the effects of
634 neurotransmitter or hormone changes are less intuitive. Therefore, we hypothesized that, if
635 a trait is adaptive, it would be present in multiple independently evolved cavefish
636 populations (69). As a proof of principle, higher starvation resistance and triglyceride
637 content (62) conform to this criterion. We tested neurotransmitter and hormone levels and
638 showed that GH and serotonin change in multiple CF concordantly to the initial SF plastic
639 response. Conversely, TSH showed the opposite direction of changes, suggesting that the
640 initial SF plastic response was not adaptive.

641 Also opposite to the expected direction of change, melanophore numbers increase in D/D
642 SF. The increase in melanophores in dark-raised SF was modest and confined to specific
643 regions of the body, but consistent with up-regulation of some melanosome-related genes in
644 the transcriptome. This result was unexpected but considered reliable, since SF x CF hybrids
645 can also show hyper-melanization (70), and neutral mutations are a likely cause of
646 melanophore reduction in *A. mexicanus* CF (71). We found a surprising result in another
647 classic troglomorphic trait, eye degeneration: although visual perception was a GO term
648 associated with down-regulated DEGs in D/D SF transcriptome, we were unable to detect
649 changes in eye size. However, D/D SF exhibited well-developed retinal layers, and we even
650 recorded thickening of the inner and outer nuclear layer, and the photoreceptor layer. These
651 regions are cell proliferation and intercalation centers so it is possible that darkness activates
652 mitotic activity in these retinal layers as a compensation for defective transmission of light
653 from the lens and induced retinal stress (72). Our results are consistent with studies showing
654 teleosts change body pigmentation and retinal phenotypes in response to light levels (73–
655 75). For example, *Astyanax* F2 hybrids with small or absent eyes also show
656 hyperpigmentation, which has been explained as background adaptation due to the lack of
657 visual input (76). Overall, our results suggest that plasticity lacks the propensity to produce
658 adaptive variants, and non-adaptive, even maladaptive, effects seem to be as likely as
659 adaptive outcomes.

660 Most of the plastic traits still show sensitivity to darkness in CF (e.g. GH, starvation
661 resistance, metabolic rate; and triglyceride levels), but the magnitude of response is smaller
662 than in SF. However, some traits have completely lost plasticity in CF, such as TSH levels and
663 in particular pigmentation and eyes, which are absent in Pachón and other fully
664 troglomorphic CF. The process of replacing a plastic trait by a monomorphic trait
665 desensitized from environmental stimuli is called canalization (77). Our results suggest that
666 different traits can have different extents of canalization during the evolution of CF, implying
667 that the rate of canalization is not proportional to the time lapse since colonization of the
668 new habitat. The loss of plasticity may be a consequence of drift because a phenotype is

never expressed in a derived environment or it can be adaptive if maintenance of plasticity is costly or only one phenotype is favored in the environment (78). Importantly, the three traits that have been completely canalized in CF, showed plastic response in SF that was non-adaptive or potentially maladaptive. This suggests that natural selection acting against plasticity was involved in the evolution of these traits in CF. A novel aspect of the evolution of classical troglomorphic traits, the reduction of eyes and pigmentation, may be that they are a consequence of strong selection against non- or mal-adaptive plastic responses during colonization of caves by surface ancestors.

To test if the inheritance of some of the plastic phenotypic changes described here occur during the next generation, we induced spawning in D/D SF and investigated starvation resistance and metabolic rate in the offspring (dSF), traits shown to exhibit plasticity in the parental D/D generation. Neither of these traits showed differences between offspring spawned in the light or dark when maintained in L/D. However, when maintained in D/D, dSF showed continued and enhanced plasticity of both traits, as well as a potential adaptive-tuning of the character states, suggesting that plasticity is evolvable and can be an evolutionary adaptation itself. Increased plasticity was previously suggested to be an important stepping stone in the process of adaptations to extreme environments (52,79). Therefore, consistent with the Baldwin effect (80), the evolution of cave related traits may have proceeded through initial selection for continued plasticity and refinement of the characters, followed by selection of individuals carrying the most improved variants of the phenotype. Higher growth hormone and triglyceride levels, as well as lower body serotonin levels in PA than D/D SF, also imply refinement of the initial SF plastic response in CF. In nature SF are periodically swept into caves and plasticity in many traits that enable survival may be critical to extend their lifespan long enough to leave offspring. According to our results, the next generation may be slightly better equipped for coping with the cave environment by increased plasticity and small adaptive switches in traits.

One of the reasons the role of plasticity in the adaptive evolution has been contested is its randomness in the production of outcomes where non-adaptive and mal-adaptive responses are as likely as adaptive responses. However, our study suggests that even the initial non-adaptive plastic responses may eventually produce an adaptive outcome. Some traits, including the hallmarks of troglomorphic adaptations (the loss of eyes and pigmentation) initially show non-adaptive plastic responses but the complete canalization of the opposite character state in CF indicates strong selection against plasticity in SF colonizers. Non-adaptive plasticity may have a greater role in evolution than previously appreciated because it may increase the strength of selection against plasticity, and perhaps, in some cases, may affect the whole trait itself. Conversely, in cases where plasticity produces adaptive outcomes, initial selection will act to enhance plasticity, and plasticity will be maintained in the derived lineage for many generations. Overall, plasticity has a critical role in producing variability (adaptive or not) on which selection can act (for or against) to produce individuals with optimal fitness in the new environment.

709 We also uncovered two cases in which the plasticity of a single trait changes within an
710 individual over the course of development. Both metabolic rate and serotonin showed shifts
711 in plasticity depending on developmental stage. Metabolic rate initially (at 2.5 dpf) changes
712 in response to the light regime but by 7.5 dpf this response is lost in PA. In contrast,
713 serotonin levels are plastic in adults but not in 7 dpf larvae. Interestingly, switches in
714 plasticity occur at the same time as switches in character state. At 2.5 dpf there is no
715 difference in metabolic rate between SF and PA, but by 7.5 dpf, the metabolic rate in PA is
716 lower than in SF, agreeing with previous studies done on adults (63,64). In the case of
717 serotonin, character states show opposite relationships at different stages: they are lower in
718 larvae but higher in adult SF compared to PA. Therefore, for metabolic rate and serotonin
719 levels, both the trait and its plasticity change concordantly, suggesting that the underlying
720 mechanisms of character development may also influence the ability to respond to
721 environmental stimuli. These two examples show that plasticity is not an intrinsically fixed
722 property of a trait but can develop by itself. In our examples, plasticity develops
723 concordantly with the traits it affects, and it can develop in a direction of expansion or
724 reduction. The development of metabolic rate in PA is accompanied by canalization of the
725 same trait. In the case of serotonin, development of the 5-HT system is accompanied by an
726 increase in its plasticity.

727 Phenotypic plasticity can fill several gaps in the current model of cave colonization and the
728 evolution of cave dwelling organisms. Our study shows how a colonizing surface ancestor,
729 having no adaptations to caves and presented with the challenges of low food availability,
730 orientation, and reproduction in complete darkness, could have overcome a transition to the
731 extreme subterranean environment and establish a cave-adapted lineage. Further, due to
732 plasticity it is possible to evolve adaptations within a very short timeframe; in fact,
733 numerous adaptive traits can arise within a single generation in response to only one, albeit
734 the most relevant, environmental cue: darkness. Finally, plasticity may enable the
735 maintenance of distinct phenotypes in the face of gene flow from ancestral surface-dwelling
736 populations. The incorporation of phenotypic plasticity as one of the mechanisms underlying
737 cave colonization and adaptive evolution provides a solution to dilemmas associated with
738 drift or selection (81) acting alone to induce cave-related phenotypes in *A. mexicanus*.
739 Because multiple successful transitions by the same surface ancestors are not unique to
740 *Astyanax* (82,83), some animals groups have colonized caves repeatedly in different times
741 and geographical regions, and several unique cave dwellers are closely related to highly
742 invasive (and plastic) surface dwelling species (84,85), it is possible that plasticity may be a
743 general phenomenon in the colonization and adaptation to cave environments.

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746 **COMPETING INTERESTS**

Authors declare no competing interests.

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