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The first cavefish in the Dinaric Karst? Cave colonization made possible by phenotypic plasticity in *Telestes karsticus*

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ABSTRACT

Cave animals are an excellent model system for studying adaptive evolution. At present, however, little is known about the mechanisms that enable surface colonizers to survive in the challenging environment of caves. One possibility is that these species have the necessary genetic background to respond with plastic changes to the pressures of underground habitats. To gain insight into this process, we conducted a comparative study with the fish species *Telestes karsticus*, which occurs in a hydrological system consisting of an interconnected stream and a cave. Results showed that *T. karsticus* resided year-round and spawned in Sušik cave, making it the first known cavefish in the Dinaric Karst. Cave and surface populations differed in morphological and physiological characteristics, as well as in patterns of gene expression without any evidence of genetic divergence. To test whether observed trait differences were plastic or genetic, we placed adult fish from both populations under light/dark or constant dark conditions. Common laboratory conditions erased all morphometric differences between the two morphs, suggesting phenotypic plasticity is driving the divergence of shape and size in wild fish. Lighter pigmentation and increased fat deposition exhibited by cave individuals were also observed in surface fish kept in the dark in the laboratory. Our study also revealed that specialized cave traits were not solely attributed to developmental plasticity, but also arose from adult responses, including acclimatization. Thus, we conclude that *T. karsticus* can adapt to cave conditions, with phenotypic plasticity playing an important role in the process of cave colonization.

Keywords: Maladaptive and adaptive phenotypic plasticity; Troglobionts and stygobionts; Endemic leuciscid fish; Acclimatization; European cavefish; Cave adaptations; Gene expression differences

INTRODUCTION

The precise mechanisms enabling certain species to colonize novel environments remain elusive. The first biological responses of organisms after entering caves can provide important insights into this process, as the colonization of caves is one of the most extreme habitat changes on Earth, involving transition from a light- and nutrient-rich environment to one under constant darkness and nutrient scarcity. It also involves the transition from highly biodiverse habitat to an environment where only a few highly specialized organisms are present (Mammola, 2019). Unique traits that have convergently evolved in obligate cave dwellers include the loss of pigmentation, loss of eyesight, metabolic adaptations, and behavioural and circadian changes (Culver & Pipan, 2014, 2019). How did the surface-dwelling animals that originally colonized caves cope with the environmental challenges that took generations for subterranean species to adapt to? In other words, how did they manage to survive and reproduce in caves without any specialized adaptations? In some cases, such as the Mexican tetra (*Astyanax mexicanus* (De Filippi 1853)), cave adaptations evolved within a remarkably short time, despite ongoing gene flow from rivers with large populations of surface fish to caves with much smaller populations of cavefish (Herman et al., 2018). A recent study examining an *Astyanax* population in Texas revealed notable phenotypic distinctions between cave-dwelling fish and those residing in a stream, despite the relatively recent introduction of *Astyanax* to Texas and the cavefish population having an estimated age of less than 100 years (McGaugh et al., 2020). Bilandžija et al. (2020) showed that phenotypic plasticity induced by exposure to total darkness can cause a plethora of trait changes in surface fish, even within a single generation, most of which resemble the adaptations of cavefish. *Astyanax* surface fish in darkness exhibit lower

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metabolic rates, higher starvation resistance, higher fat content, changes in the endocrine system, and serotonin levels similar to those in cavefish. The same *Astyanax* surface fish in darkness also show increased pigmentation and proliferation in the retina, as examples of non- or even maladaptive plasticity, given that cavefish completely lack pigment and eyes (Bilandžija et al., 2020). Here, we refer to traits that have evolved convergently in cave-dwelling animals as adaptive, without presupposing their effects on fitness or the involvement of natural selection in their evolution, both of which are largely unknown. This is in contrast to maladaptive changes in traits that behave opposite to optimal phenotypes in caves.

Darkness is known to be the only environmental condition common to all subsurface habitats (Culver & Pipan, 2019; Howarth & Moldovan, 2018), and induces several plastic changes in *Astyanax* (Blin et al., 2018; Carlson & Gross, 2018; Moran et al., 2014; Rasquin, 1949; Rasquin & Rosenbloom, 1954; Sears et al., 2020; Tanvir et al., 2021; Yoffe et al., 2020; Yoshizawa et al., 2010) and other fish (Riesch et al., 2016). Studies comparing closely related cave-surface animals, including salamanders (Guillaume et al., 2020; Manenti et al., 2013), isopods (Horváth et al., 2021), and amphipods, from the same hydrological systems (MacAvoy et al., 2016; Pacioglu et al., 2020) suggest that plasticity plays an important role in the response of a species to a new habitat. However, it is not known whether a high degree of plasticity is critical for all cave colonizers to ensure successful colonization of caves.

Although cavefish are the most species-rich vertebrate group found in caves (Culver & Pipan, 2019; Niemiller et al., 2019), and the Dinaric Karst is recognized as a hotspot of subterranean biodiversity (Culver & Sket, 2000; Deharveng et al., 2012), there are no documented cavefish species in this area. This is surprising given the high biodiversity and endemism of freshwater fish in the surface waters of the Dinaric Karst in Croatia (Ćaleta et al., 2019). While several fish species in genera *Delminichthys* Freyhof, Lieckfeldt, Bogutskaya, Pitra & Ludwig 2006, *Phoxinellus* Heckel 1843, and *Telestes* Bonaparte 1837 are known to reside in Dinaric Karst caves during low-water periods when many surface streams dry up, they reemerge and resume their surface lifestyles as soon as water level conditions become more favorable (Mrakovčić et al., 2006). Several of these species actively migrate via subterranean water conduits (Buj et al., 2022; Palandačić et al., 2012), but there are no reports of any of these fish reproducing in caves. Moreover, Reier et al. (2022) claimed that two *Delminichthys* species mostly stay underground but emerge to the surface to spawn.

The karstic dace (*Telestes karsticus* Marčić & Mrakovčić 2011) is a small endemic leuciscid fish found in only a few locations in Croatia (Ćaleta et al., 2019; Marčić, 2013; Marčić et al., 2011). To date, studies on this species have primarily focused on its age, growth, reproductive biology, and seasonal diet profile (Marčić et al., 2021, 2017a, 2017b). *Telestes karsticus* is found in abundance in the Sušik stream, with an estimated population size of more than 10 000 individuals (Marčić et al., 2011). This species is also frequently observed in the Sušik cave (Butorac et al., 2019; Branko Jalžić, pers. comm.), a large sinkhole cave where the Sušik stream disappears underground.

Morphologically, fish in Sušik cave strongly resemble those in the surface stream and show no obvious cave adaptations. Here, we describe some of the natural history aspects of the cave population, as well as its genetic relationship with and phenotypic differences to the surface population. We tested whether the presence of *T. karsticus* in extremely different environments is a result of genetic divergence between populations due to local adaptation or due to plasticity. To test this, we exposed fish from both populations to constant dark and light/dark conditions. We predicted that if phenotypic variability between fish from the cave and stream is caused by plasticity, then trait differences would disappear in laboratory groups maintained under identical conditions. However, if the changes have a genetic component, no differences would be expected between fish in the wild and laboratory and observed differences between cavefish and stream fish would remain the same.

MATERIALS AND METHODS

Study species and site

Two populations of *T. karsticus* were included in the study: one from Sušik stream (surface fish) and one from Sušik cave (cavefish) (Figure 1A–C). Sušik stream (N45.145676; W15.078079, WGS84) flows through the small karst field polje Lug on Velika Kapela Mountain in Croatia. The stream sinks about 70 m before the entrance to Sušik cave (N45.145417; W15.09015, WGS84) (Figures 1D, E), and reappears in the cave as a small stream. Sušik cave is 1 829 m long and the underground stream ends in a sump (Butorac et al., 2019). During extremely high water levels, the surface stream reaches the cave through the main entrance. In the dry season (usually in summer), the surface stream dries up almost completely, leaving only a few pools that serve as refuges for the fish.

Ethics statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were strictly followed. All animal collection protocols complied with the current laws of Croatia. All field research, sampling, and laboratory procedures were approved by the Croatian Ministry of Environmental Protection and Energy.

Tagging procedures and population estimates

We used the capture-mark-recapture method to estimate population sizes. Fish were captured using a backpack electrofishing device (Hans-Grassl, model IG-200-1), then weighed, photographed, tagged, and released (except in April 2020 when fish were brought to the laboratory). At the surface, fish were captured along a 200 m transect covering all habitats with the same effort (1 h) during each sampling event. There were seven sampling events (20 July 2020, 21 July 2020, 8 April 2021, 28 April 2021, 15 June 2021, 13 September 2021, and 11 November 2021), and fish were tagged on 20 July 2020, 28 April 2021, and 13 September 2021. Only fish with a total length greater than 40 mm were counted and tagged. In the cave, fish were captured in the shallow parts of the cave downstream from the entrance towards the sump, for a total of 200 m, with the same effort (1 h) at each sampling event. Fish were anesthetized for tagging with 0.25 g/L tricaine methanesulfonate (Sigma-Aldrich, cat. #A5040, USA). We injected dye from a Visible Implant Elastomer (VIE) Tags hand injection kit (Northwest Marine Technology Inc., cat. #K0273200, USA) near the operculum, using different colors for cavefish and surface fish. Statistical analyses were performed based on the Petersen

Figure 1 Map of the study area, entrance to Sušik cave, and photos of *Telestes karsticus* adults and embryos and other species observed in Sušik cave

A–C: Individuals of *T. karsticus* from Sušik stream (A) and Sušik cave (B, C) (Scale bars: 10 mm). D: Map of Polje Lug karst field and sampling sites. E: Entrance of Sušik cave. F: Pre-hatched embryonic stages of *T. karsticus* found in Sušik cave in May 2022. G–I: Observed non-cave-dwelling animals found in Sušik cave: *Asellus aquaticus* (G), *Austropotamobius torrentium* (H), and dytisciid beetle (I). Photo by: Jana Bedek (A,B,E,G,H,I)); Branko Jalžić (C); Marko Lukić (F).

method (Krebs, 1999) with default settings, using Ecological Methodology v7.0 software. Data used for analysis were obtained from the 20–21 July 2020 sampling trip, ensuring compliance with the closed population requirements of the method. During this sampling period, the transect where surface fish were sampled was separated from the rest of the stream by natural and artificial barriers, preventing fish migration during low water levels. Additionally, we confirm that no floods occurred between the capture and recapture episodes.

Laboratory experimental conditions

Fish from both the cave (CF, *n*=60) and stream (SF, *n*=60) were brought to the laboratory in April 2020 and randomly split into complete darkness (DD) and light/dark cycle (LD) conditions, resulting in four experimental groups: two under complete darkness (SFDD and CFDD) and two under control light/dark conditions (SFLD and CFLD). Each group contained 30 fish at the beginning of the experiment, which were maintained at 21–22 °C in 120 L tanks with filters and aerating stones. Fish were kept on the same diet: extruded fish food (S.A.K. 55 granules, EXOT HOBBY, cat. #102124, Czech Republic) and frozen red mosquito larvae (Petra Aqua, cat. #Y306, Czech Republic). Fish were fed manually or by automatic feeders (model AF2005D, Resun, China). One third of the water was changed weekly and aquaria were cleaned of food debris and feces daily. Dim red light was used for handling fish in the dark. After 7 months in the common garden experiment, fish were anaesthetized and photographed for morphometric analyses, with three individuals from each group sampled for RNA sequencing (RNA-seq). After 22 months, at the end of the experiment, a total of 30 surviving fish were euthanized for final phenotyping (*n*: SFLD=9; SFDD=6; CFLD=6; CFDD=9), which included morphometry and quantification of pigmentation and lipid content in the liver.

Phenotyping

Fish were photographed with a scale using an Olympus TG5 mounted on a tripod. A total of 12 variables (Supplementary Figure S1A) were measured using ImageJ software (National Institute of Health, USA): standard length, eye diameter (anteroposterior and dorsoventral axis of eye), head (head length, maximum head height and dorsal head length), humpback, caudal fin length (dorsal and ventral lobe), and body height at three points (end of operculum, maximum height, and height at base of ventral fin). All measurements were taken as a straight line from point to point, except for the humpback measure, which was measured as surface area. We calculated Fulton's fitness coefficient *K* using the formula from Anderson & Neumann (1996).

For pigmentation analysis, fish were photographed in water to minimize glare and on a standardized background for normalization using a Canon EOS 80D digital camera (Japan). Settings were set to: ISO 100, F11, s 1/160; RAW + L Jpeg; flash for white balance; Macro Canon MP-E 65 mm (1×); Canon Speedlite 580EX II Flash (manual ¼, two diffusers). The mean gray value was obtained in ImageJ using a polygon near the operculum and around the lateral line (Supplementary Figure S1B) and was normalized with the mean gray value of the background. To test whether this method is robust and reliable for inferring possible changes in

pigmentation between the two fish morphotypes, we performed an additional analysis by counting melanophores in the same area used to determine mean gray levels in each of 10 randomly selected surface and cavefish. Eyes from 10 wild surface fish and 10 cavefish were dissected, embedded in paraplast, sectioned, and stained with hematoxylin and eosin, as described in Bilandžija et al. (2020). Images were taken with a digital camera (Carl Zeiss Microscopy GmbH, Germany) at 40× magnification, while eye diameter was measured at 2× magnification. Images were processed and analyzed using ZEN v3.4 (blue edition) software (Carl Zeiss, Germany). The thickness of the whole retina and seven different layers (ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, photoreceptor cell layer, retinal pigment epithelium) were measured on 10 randomly chosen fields from each sample. Retinal layer thickness was normalized by eye diameter.

Lipids were extracted from dissected livers following the method of Bligh & Dyer (1959), and lipid concentrations were calculated according to the standards of tripalmitin in chloroform. Absorbance was measured spectrophotometrically at a wavelength of 340 nm (Infinite M200, Switzerland). We analyzed a total of 22 liver samples from wild *T. karsticus* (11 surface fish and 11 cavefish) and 28 liver samples from laboratory fish (*n*: SFLD=8; SFDD=5; CFLD=6; CFDD=9).

RNA extraction and sequencing

Three fish from each group were homogenized in liquid nitrogen with either a mortar and pestle for wild fish or with cryogenic 6875 Freezer/Mill (SPEX SamplePrep, USA) for laboratory cohorts after 7 months in experimental conditions. Total RNA was extracted from whole-body homogenates using a SPLIT RNA Extraction Kit (Lexogen, cat. #008, Austria). RNA quality was evaluated by gel electrophoresis, spectrophotometry, and Bioanalyzer analysis (Agilent, USA). RNA samples were treated with TURBO DNAse (Thermo Fisher Scientific, cat. #AM2239, USA) until there was no amplification of the *cytb* gene. Polymerase chain reaction (PCR) was performed with Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, cat. #M0531, USA) using primers GluF and ThrR (Zardoya & Doadrio, 1998), with 30 s of initial denaturation at 98 °C, followed by 30 cycles at 98 °C/10 s, 55 °C/20 s, and 72 °C/25 s, and a final extension at 72 °C for 7 min. Samples were sent for sequencing to Novogene Europe (Cambridge, UK): 800 ng of RNA from laboratory samples for nondirectional sequencing and 1 600 ng of RNA from wild samples for directional sequencing. Libraries were prepared using library prep kits (New England Biolabs, USA) and Illumina PE150 sequencing was performed on the NovaSeq 6000 platform (Illumina, USA).

***De novo* transcriptome assembly, annotation, and differential expression analysis**

De novo transcriptome assembly was performed using the Galaxy Europe platform (https://usegalaxy.eu/) (Galaxy Community, 2022) following the pipeline reported in Bretaudeau et al. (2021) and Batut et al. (2018). Trimmomatic v0.38 (Bolger et al., 2014) was used to filter low-quality reads and FastQC v0.11.9 (Andrews, 2010) or MultiQC v1.11 (Ewels et al., 2016) was used for quality check. Trinity (Grabherr et al., 2011) was used for assembly, with a minimum contig length of 300 bp and minimum count for *K*-mers of 3. To filter low-expressed transcripts (all isoforms above a dominant expression of 1% were retained), we first remapped the raw reads using the Align reads and estimate abundance tool, with RSEM for abundance estimation and Bowtie for alignment. The build expression matrix tool was used to merge mapping tables and compute normalizations. Assembly quality was assessed using BUSCO (Simão et al., 2015), with the lineage set to *Actinopterygii*. For annotation, we used Diamond v2.0.15 (Buchfink et al., 2015) BLASTX and BLASTP with the ncbi_nr_2021_01 reference database. TransDecoder (Haas et al., 2013) was used to identify candidate coding regions and TMHMM v2.0 (Cock et al., 2013) was used to find transmembrane domains in the protein sequences. Trinotate (Grabherr et al., 2011) was used for automatic functional annotation with the help of output from Hmmscan (Finn et al., 2011). To identify differentially expressed genes (DEGs), remapping of the filtered transcriptome was performed using the Align reads and estimate abundance tool, after which the mapping tables were merged and Trimmed Mean of M-values (TMM) normalization was computed using the Build expression matrix tool. Next, differential expression analysis was performed for raw counts using DESeq2 (Love et al., 2014). To extract and cluster differentially expressed transcripts, the *P*-value cutoff for the false discovery rate (FDR) was set to 0.001. We wrote the script using the Biopython Esummary tool (Cock et al., 2009) to retrieve summaries against "sprot_Top_BLASTX_hit" IDs in Python v3.10.7 with the Jupyter Notebook. We used REVIGO (Supek et al., 2011) to remove redundant Gene Ontology (GO) terms and visualize them. TopGO was used for gene set enrichment analysis (Alexa & Rahnenfuhrer, 2022). All raw sequences were submitted to NCBI, SRA (BioProject ID: PRJNA914542; accession numbers: SAMN32340189–SAMN32340206).

Variant calling and genetic differentiation between cave and surface populations

Variant calling was performed using several different tools on the Galaxy Europe platform. We used Trinity assembly of the wild samples, followed by filtering of low-expressed transcripts. To obtain a genome-like reference as a substrate for identifying allelic polymorphisms, we first ran CD -hit (Galaxy v4.8.1) for clustering (Fu et al., 2012; Li & Godzik, 2006), then generated SuperTranscripts (Galaxy v2.9.1) from assembly (Davidson et al., 2017; Grabherr et al., 2011). Finally, we filtered the transcripts to retain only those >1 000 bp in length. To match the reads against a reference transcriptome, BWA-MEM2 (Galaxy v2.2.1) was used (Li, 2013; Li & Durbin, 2010), while subsequent statistics in the BAM index files were checked with SAMtools idxstat and SAMtools flagstat (Li, 2011a, 2011b; Li et al., 2009). To generate a VCF file containing genotype probabilities from BAM alignment, the bcftools mpileup tool (Galaxy v1.15.1+galaxy2) was used. Finally, single nucleotide polymorphism (SNP) variant calling from VCF, excluding indels, was performed using bcftools call (Galaxy v1.9+galaxy1) (Li et al., 2009). For filtering, VCFfilter (Galaxy v1.0.0_rc3+galaxy3) was set to --info-filter to remove alleles that did not pass the filter (DP>10 and DP<250; QUAL>20).

Conversion of the filtered VCF file to an Arlequin .arp file was performed with PGDSpider v2.1.1.5, while analysis of molecular variance (AMOVA) and FST were performed with Arlequin v3.5.2.2 (Excoffier & Lischer, 2010).

Statistical analyses

All statistical analyses, except for multivariate analysis of variance (MANOVA), were performed using Python v3.10.7 in

Jupyter Notebook. We first checked normality with Q-Q Plot. Independent sample *t*-tests, either student or Welch's *t*-test based on Levene's test for equality of variances, were used for comparison of the two groups of wild *T. karsticus*. For analyses of the four laboratory groups, Welch's analysis of variance (ANOVA) was used if the assumption of homogeneity of variance was violated, or classic one-way ANOVA was used if variances were homogenous. Tukey's *post-hoc* test was used, with the significance level set to $P<0.05$. To examine the differences in morphometric variables within and between all groups, MANOVA was performed. MANOVA is used to compare multivariate sample means and addresses the inflated type 1 error in ANOVA by considering all dependent variables simultaneously as well as their intercorrelations. Appropriate *post-hoc* tests, either Scheffe or Games-Howell, were performed, with $P>0.05$ considered as statistically significant. Assumption, MANOVA, and *post-hoc* tests were analyzed using SPSS v26.0 (IBM Corp, USA), while graphs were generated with R v4.2.2 (R Foundation for Statistical Computing, Austria). All details of statistical results are listed in Supplementary Tables S1, S2. In the box-whisker plots, the middle black line represents the median, limits of the colored boxes represent quartiles, and vertical black lines extend to the minimum and maximum value of the trait. Outliers are depicted as diamonds.

RESULTS

Size of permanent *T. karsticus* population in Sušik cave

From April 2020 to November 2022, we visited Sušik cave a total of 13 times. Fish were observed on every occasion, indicating that *T. karsticus* inhabits the cave year-round. In April 2021, we also observed gravid females and spawning males. In May 2022, we organized a specific field trip to determine whether fish were spawning in the cave. On that occasion, we found reproductive adults as well as developing eggs and pre-hatched embryos (Figure 1F) within Sušik cave, in the shallow part of the underground stream with a rocky bottom and strong current, similar to the surface habitat where fish spawn. This suggests that the *T. karsticus* fish can reproduce in the cave. In addition to *T. karsticus*, many other surface-dwelling animals were regularly recorded in Sušik cave, which were likely washed in by floods during high water levels, including *Austropotamobius torrentium* (Schrank, 1803), *Bufo bufo* (Linnaeus, 1758), *Ancylus* sp. O. F. Müller 1773, *Synurella* sp. Wrześniowski 1877, Dytiscidae beetles, caddisflies, Trichoptera larvae, and a population of cave-like *Asellus aquaticus* (Linnaeus, 1758) (Figure 1G–I).

We estimated the population sizes of *T. karsticus* in Sušik stream and cave and tried to confirm migration between the surface and cave using the repeated capture-mark-recapture method. In total, 204 individuals from the cave and 303 individuals from the stream were tagged and released, with no casualties, on seven occasions spanning a total of 14 months. For population size estimates, we used the 20–21 July sampling event as the conditions were favorable to meet the closed population requirement of the method. According to the original Petersen estimate, the number of cavefish was estimated at 163.5 (95% CI=145.0–189) and the number of surface fish in the 200 m section of the stream was estimated at 1 509 (95% CI=944.3–2 749.5). Throughout the 14-month period, no marked cavefish were found outside the cave, nor were any marked surface fish recaptured inside the cave.

Phenotypic differences between wild cavefish and surface fish

Telestes karsticus individuals in the cave and stream showed numerous morphometric differences. The standard length of the surface fish was significantly smaller than that of the cavefish ($P<0.001$), although the head, measured at three different points, was larger ($P<0.001$) (Figure 2A–C). Body height of cavefish was smaller at the operculum ($P<0.001$) (Figure 2D) but larger more caudally ($P<0.001$) (Figure 2E) and humpback surface area was also larger ($P<0.001$) (Figure 2F). Only two traits related to the caudal fin did not differ significantly between the two morphs ($P>0.05$) (Figure 2G, H).

Eye diameter was significantly smaller in cavefish, regardless of the axis measured ($P<0.001$) (Figure 2I). However, most retinal layers were thicker in cavefish than in surface fish. Only the retinal pigment epithelium was thicker in surface fish ($P<0.001$). Three of the remaining six layers were significantly larger in cavefish: inner nuclear layer ($P=0.01$), outer nuclear layer ($P=0.014$), and photoreceptor cell layer ($P<0.001$) (Figure 3A).

Cavefish were significantly heavier than surface fish ($P<0.001$) (Figure 3B) and had a higher Fulton body index ($P<0.001$) (Figure 3C). The overall fat content in the liver was significantly higher in cavefish ($P=0.033$; Figure 3D). Cavefish also had lighter pigmentation ($P=0.036$) (Figure 3E) and a lower number of melanophores (Supplementary Figure S1C) than surface fish.

Phenotypic differences between laboratory-raised fish

To test whether phenotypic differences between cave and surface *T. karsticus* in the wild arose through phenotypic plasticity or local adaptation, we exposed cavefish and surface fish to complete darkness and light/dark conditions in a common garden experiment. If a trait is plastic, the differences between wild populations are expected to disappear in the laboratory environment. MANOVA of morphometric traits showed significant differences between the groups (Pillai's Trace, $P<0.001$; Figure 2A–I). After 7 months in the common garden experiment, some differences between morphs were still present, including body height and length of the dorsal lobe of the caudal fin, but all differences disappeared by 22 months (Figure 2D, E, G). Comparisons between wild fish and common garden experiment fish showed that most traits in the surface fish changed in response to laboratory maintenance. In cavefish, no differences were observed between wild and laboratory fish regarding humpback area (Figure 2F), dorsal head length, and head height (Figure 2B, C). Eye size was only different between the wild cavefish and the CFDD2 group (Figure 2I), whereas differences in height at the operculum, head length, and ventral lobe of the caudal fin were present at 7 months but not at 22 months under the experimental conditions (Figure 2D, A, H, respectively). There were no significant differences between fish maintained under complete darkness vs. light/dark conditions, except in the width and dorsal lobe of the caudal fin in surface fish at 7 months (but not at 22 months) of the experiment. In addition to morphometric traits, we found significant differences in lipid content between SFLD and SFDD ($P=0.007$) and between SFDD and CFDD ($P=0.008$) (Figure 4A). We also observed significant differences in pigmentation level ($P=0.039$), with Tukey's *post-hoc* test revealing a significant difference between SFLD and SFDD ($P=0.038$) (Figure 4B).

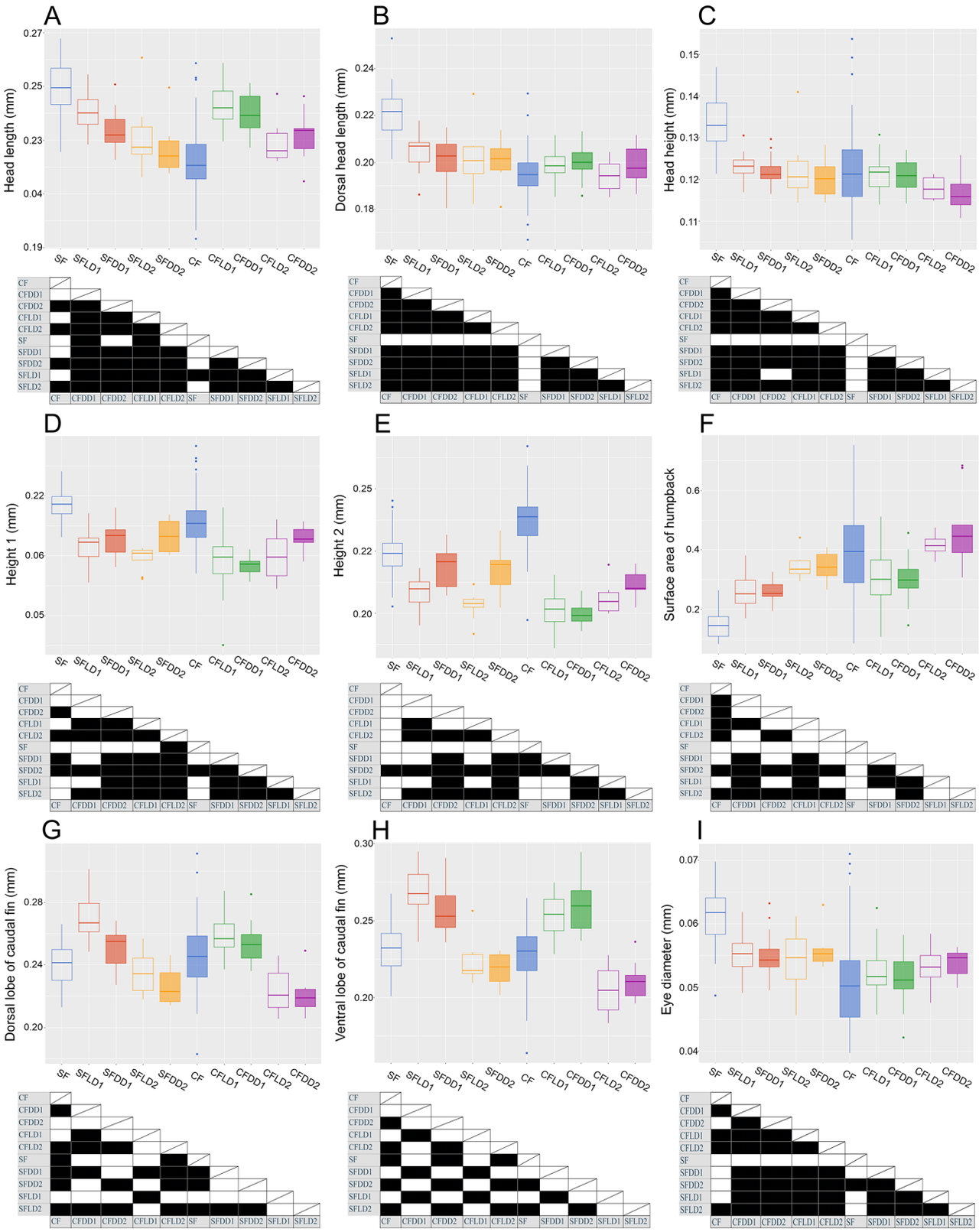

Figure 2 Morphological differences between wild *Telestes karsticus* surface fish (SF) and cavefish (CF) (*n*: SF=67, CF=60) , and laboratory fish of both morphs maintained in complete darkness (DD) and control light dark conditions (LD) after 7 months (1) (*n*: SFLD1=22, SFDD1=15, CFLD1=21, CFDD1=22) and 22 (2) months (*n*: SFLD2=9, SFDD2=6, CFLD2=6, CFDD2=9) in the common garden experiment

All the morphometric variables were normalised by standard lenght. A: Head length. B: Dorsal head length. C: Head height. D: Body height at the end of operculum. E: Maximum body height + body height at the base of ventral fin/2. F: Surface area of the humpback. G: Dorsal lobe of caudal fin. H: Ventral lobe of caudal fin. I: Eye diameter (diameter along anteroposterior eye axis + diameter along dorsoventral eye axis/2). Corresponding tables with *P*-values representing statistically significant pairs (white bars) can be found below each graph.

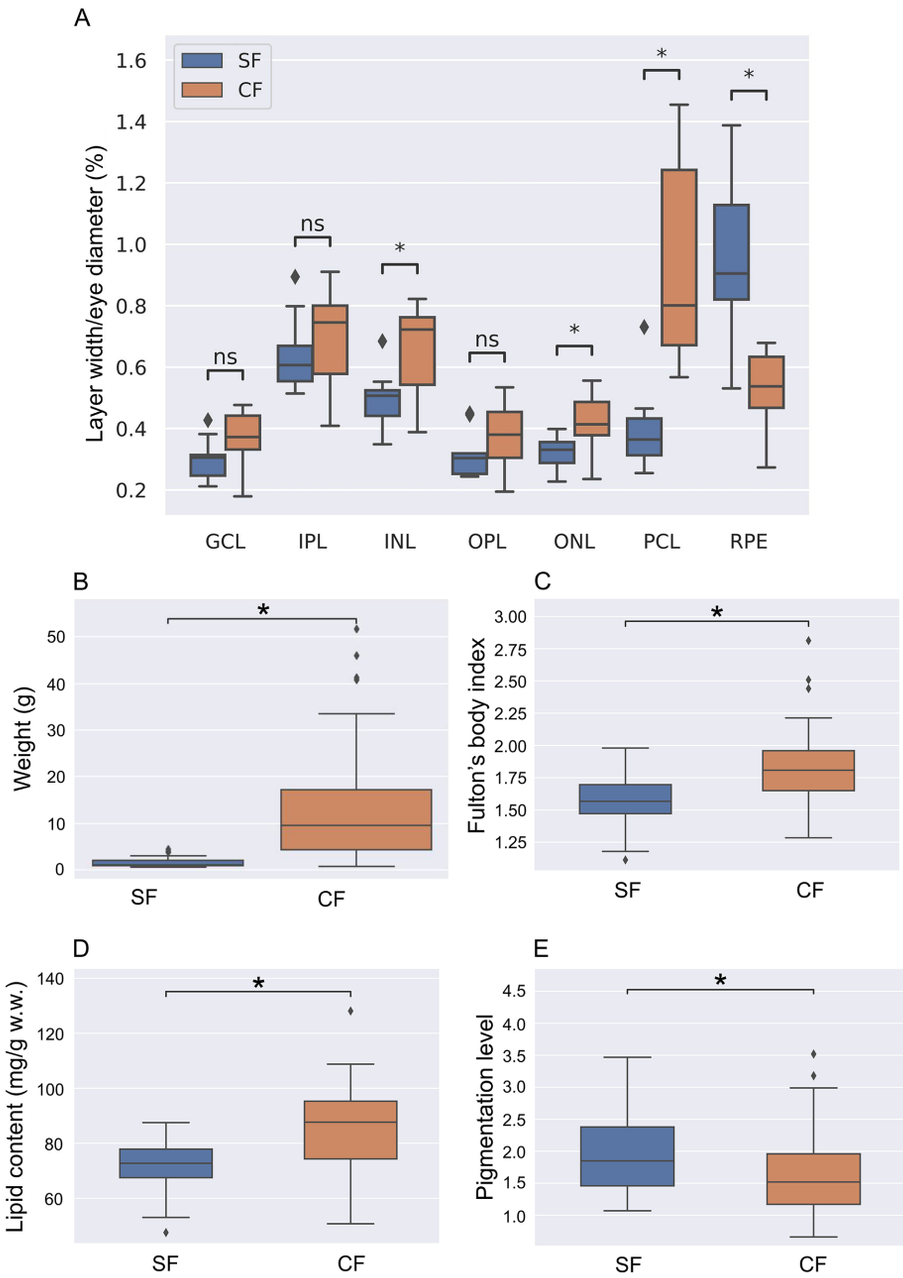

Figure 3 Phenotypic differences between wild *Telestes karsticus* from Sušik stream (SF) and cave (CF)

A: Width of retinal layers normalized by eye diameter (*n*: SF=11, CF=11): Ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptor cell layer (PCL), and retinal pigment epithelium (RPE). B, C: Weight (B) and Fulton body index (C) (*n*: surface fish=67, cavefish=60). D: Mean concentration of lipids from livers (*n*: surface fish=11, cavefish=11). E: Pigmentation level normalized by background (*n*: SF=57, CF=48). Independent sample *t*-test: ns: Not significant; *:$P<0.05$.

Gene expression changes in wild and laboratory groups of *T. karsticus*

Between 78 and 97 million reads were generated from six fish taken directly from the wild (three cavefish and three surface fish; Supplementary Table S3). DESeq analysis identified 2 870 genes as differentially expressed, most of which were up-regulated in cavefish (84.15%) (Figure 5B; Supplementary Table S4). GO terms were assigned to 483 DEGs, including visual perception, lipid and carbohydrate metabolism, gluconeogenesis, keratinocyte development, steroid synthesis, glutathione synthesis, heme oxidation, neuropeptide and dopamine receptor signaling, immune response, oxidative stress response, gamete generation, and cellular response to ionizing radiation (Figure 5A). These GO terms resemble the expected adaptive changes of an obligate cave-dwelling organism. Of the other GO categories, functions related to (post)transcriptional and (post)translational control, chromatin rearrangements, transposition, and epigenetic processes including histone modifications and methylation, suggest major rearrangements at many different levels of molecular control in *T. karsticus* cavefish compared to surface fish in nature.

We sequenced 41–49 million reads from samples from the common garden experiment and identified 4 834 DEGs in SFDD vs SFLD, most of which were up-regulated (97.02%) (Figure 5C). In contrast, only 2 007 genes were differentially expressed in the cavefish groups in the laboratory experiment, most of which (89.74%) were down-regulated in CFDD

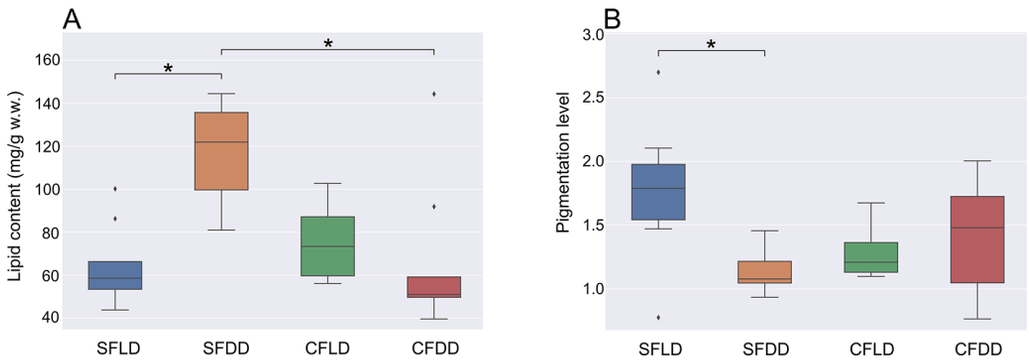

Figure 4 Phenotypic differences in *Telestes karsticus* surface fish (SF) and cavefish (CF) under constant darkness (DD) or control light/dark conditions (LD) after 22 months

A: Mean concentration of liver lipid levels in *T. karsticus* in four experimental groups (*n*: SFLD=8; SFDD=5; CFLD=6; CFDD=9). B: Pigmentation level normalized by background (*n*: SFLD=9; SFDD=6; CFLD=6; CFDD=9). ANOVA with Tukey's *post-hoc*: *: $P<0.05$.

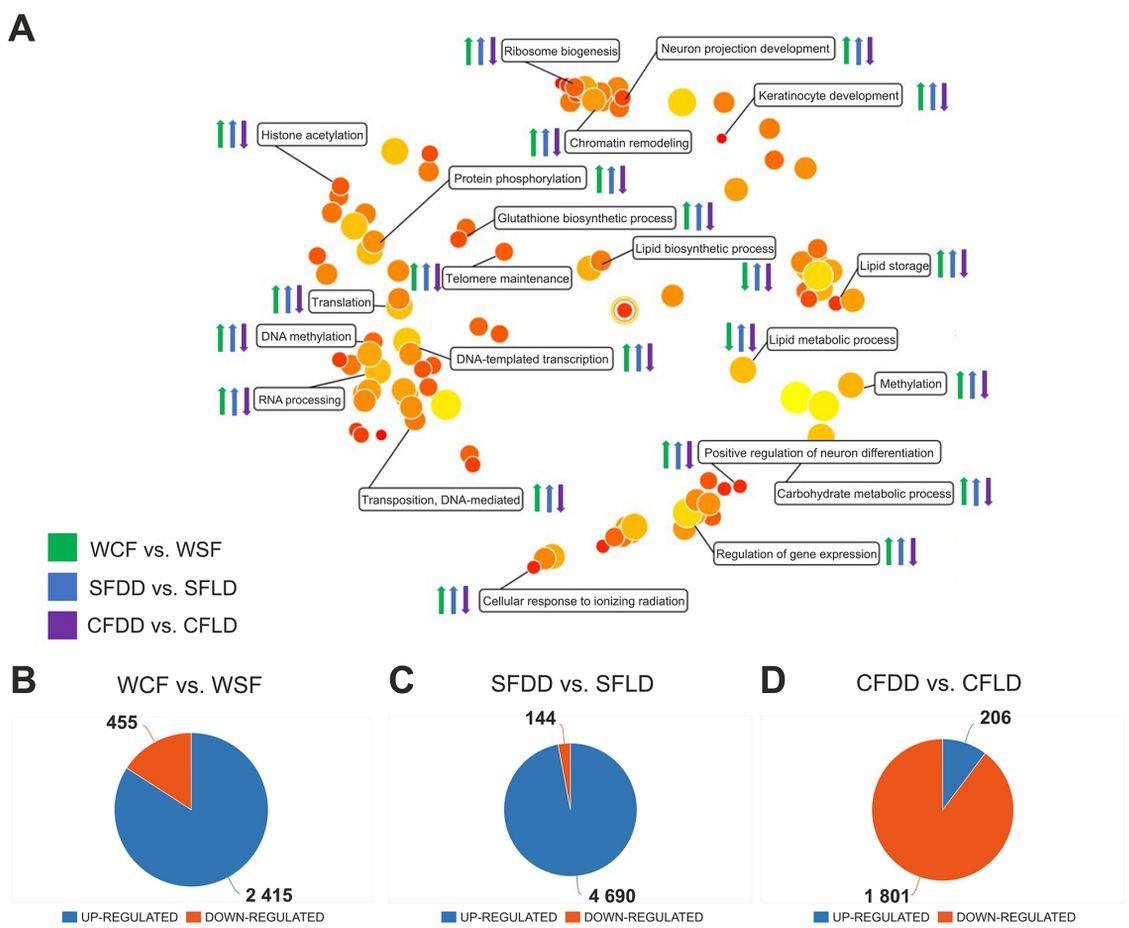

Figure 5 REVIGO chart of gene expression differences in wild *Telestes karsticus* cavefish (WCF) and surface fish (WSF) and in laboratory-maintained surface fish (SF) and cavefish (CF) raised under complete darkness (DD) or light/dark conditions (LD)

Up and down arrows indicate whether genes included in specific GO terms were up-regulated or down-regulated, respectively, in pairwise comparisons. A: Common GO terms associated with DEGs among all three group comparisons. B–D: Number of DEGs in transcriptomes of wild-caught *T. karsticus* (B), as well as common garden experimental groups of surface fish (C) and cavefish (D).

compared to CFLD (Figure 5D; Supplementary Table S5).

Next, we identified 676 DEGs in common to wild cavefish vs. surface fish and in SFDD vs. SFLD comparisons. These DEGs were involved in carbohydrate, lipid, and amino acid metabolic processes, steroid and glutathione biosynthesis, immune response, gamete generation, neuropeptide and dopamine receptor signaling, neuron projection development, (post)transcriptional control, and (post)translational control, chromatin rearrangements, transposition, and epigenetic processes, suggesting that these biological processes may have evolved in cavefish via plasticity in surface fish colonizers exposed to darkness. Most shared genes (95.56%) were in the same direction of change. In the pairwise comparisons between fish morphs in the wild and CFDD vs. CFLD, we identified fewer common genes (221), most of which exhibited the opposite direction of change. These genes were involved in metabolic, neural, and different molecular processes, such as transposition, methylation, transcription,

translation, and post-translational modifications.

According to GO enrichment analysis, DNA and microtubule binding, pseudouridine synthesis, and tRNA methylation were common to all three pairwise comparisons (Supplementary Tables S6, S7). The wild and common garden surface fish samples had several other enriched GO terms in common, including DNA duplex unwinding and replication, rRNA processing, microtubule motor activity, ATP binding, and ATP-dependent chromatin remodeler activity. In contrast, most of the enriched GO terms in cavefish from the laboratory groups were unique to these fish. This finding is similar to the pattern observed in the number of DEGs and direction of change in cavefish exposed to laboratory conditions, showing a divergent molecular signature in gene expression and regulation and further suggesting that gene expression plasticity may have changed during the process of cave colonization in cavefish (Supplementary Tables S4, S5).

Genetic differentiation between populations

To determine the genetic relationship between cavefish and surface fish populations, we used transcriptomes of all samples, including wild and laboratory groups. We identified SNPs and performed locus-by-locus AMOVA and FST. In total, 24 567 loci were analyzed. The calculated fixation index was −0.252 (P<0.001), indicating no genetic subdivision between the cave and surface populations.

DISCUSSION

Cave colonization represents an extreme environmental shift that presents various challenges to potential colonizers. Behaviorally, animals must navigate and locate mates and food without the aid of vision, while physiologically, individuals must adapt to survive in a nutrient-restricted environments (Culver & Pipan, 2014, 2019; Poulson & White, 1969; Simon, 2019). When surface and cave relatives of the same species coexist, we can observe biological differences that may be indicative of the changes involved in cave adaptation. For example, the Mexican teleost fish *Astyanax mexicanus* consists of various cave populations that have independently evolved a suite of morphological, physiological, and behavioural adaptations (Jeffery, 2020), while still residing in proximity to their closest living surface-dwelling ancestors. These populations diverged only 30–200 000 years ago (Fumey et al., 2018; Herman et al, 2018). However, the mechanisms enabling their initial cave colonization and rapid evolution of specialized cave traits remain unknown. Here, we investigated a complementary system involving the teleost fish *Telestes karsticus* from the Dinaric Karst in Croatia. These fish exist both in the stream outside the cave and within Sušik cave itself, although the cave population does not exhibit apparent adaptations specific to cave environments. Thus, this system provides an ideal opportunity to explore the role of phenotypic plasticity in the process of cave colonization.

Sušik stream is a 5 km long sinking river harboring an abundant population of *T. karsticus.* Our population estimate suggested about 1 500 surface fish within the 200 m transect of the stream. If extrapolated to the whole stream, these results are within the range of previous reports of tens of thousands of individuals (Marčić, 2013; Marčić et al., 2021). The estimated population in Sušik cave was, as expected, at least an order of magnitude smaller than that of the surface population. At least once or twice a year, water levels rapidly rise and cause floods that sweep surface animals into the cave. We found abundant and diverse surface animals in Sušik cave during most field trips, suggesting frequent immigration from the surface to the cave. Moreover, the first several hundred meters of cave passages were almost entirely occupied by surface or surface-like animals. Obligate subterranean animals (such as *Niphargus* spp.) began to appear only in deeper sections, after several hundred meters. Unlike other surface-dwelling species, *T. karsticus* was always found in the cave, regardless of the timing of flooding events. Our 14-month capture-mark-recapture survey repeatedly showed that tagged individuals did not move between habitats. No fish tagged in the cave were found outside, and vice versa. In addition, we found gravid females and ready-to-spawn males, as well as fertilized eggs firmly attached to the rocks in the cave at the same time of year that *T. karsticus* is known to reproduce at the surface (mid-spring) (Marčić et al., 2017a). It is unlikely that these developing embryos were washed from the surface because the current that would detach them from surface rocks would also destroy them before they reached the cave. In addition, it is even less likely that the eggs would reattach to the cave rocks in the same microhabitat where *T. karsticus* spawn in the surface stream, i.e., shallow part of the underground stream with strong currents. Thus, this represents the first record of a fish spawning in a Dinaric Karst cave, refuting the notion that fish in this region only use caves as refugia (Buj et al., 2022; Mrakovčić et al., 2006; Palandačić et al., 2012; Reier et al., 2022). Hence, it is likely that *T. karsticus* has established a permanent population within Sušik cave, marking the first documented occurrence of a cavefish population in the Dinaric Karst region and the second recorded instance of a cavefish population in Europe, alongside the cave loach in southern Germany (Behrmann-Godel et al., 2017).

However, it is possible that these two European fish are not obligate cave-dwellers, but only temporarily captured cave populations. They share common characteristics, such as low levels of trait specialization and immigration from adjacent surface populations. Migration from the surface may bring new genetic variants and hybridization may be crucial to generate sufficient genetic variation as a substrate for selection (Grant & Grant, 2019; Lewontin & Birch, 1966). Conversely, extensive surface immigration can have a contrasting impact on cave populations, leading to genetic and phenotypic homogeneity and constraining the adaptive evolution of these cavefish populations, ultimately reaching an equilibrium state. Of note, historical and recent gene flow (Herman et al., 2018) between surface fish and cavefish has also been demonstrated in *A. mexicanus*, suggesting that extensive migration and hybridization between these populations does not preclude genetic and phenotypic divergence. In addition, hybridization between cave and surface populations has been proposed as a mechanism for the evolution of cave-specific phenotypes in both *A. mexicanus* (Moran et al., 2022) and *A. aquaticus* (Rodas et al., 2023).

We found many phenotypic differences between the cavefish and surface fish in the wild. All studied morphometric traits, except two related to the caudal fin, differed between the cavefish and surface fish. The larger humpback area in cavefish is reminiscent of structures found in several cavefish species in the genus *Sinocyclocheilus* Fang 1936 from China (Ma et al., 2019), although *Sinocyclocheilus* humpbacks are much more prominent. Classic regressive phenotypes accompanying cave-adapted life include eye and pigmentation

reduction (Culver & Pipan, 2019; Juan et al., 2010), as also observed in the *T. karsticus* cavefish compared to the surface fish. Metabolic changes are ubiquitous in cave-dwellers, given that such environments are generally nutrient poor, with animals often relying on nutrients from sporadic floods or bat droppings (Fong, 2019). For example, *Astyanax* cavefish have evolved higher lipid storage and other metabolic adjustments to compensate for limitations in nutrient availability (Aspiras et al., 2015; Riddle et al., 2021; Xiong et al., 2018). We observed a similar trend in *T. karsticus*, where the weight, height, and body condition index were higher in cavefish than in surface fish. The large difference in weight may partially reflect the size and age structure of the population. Both surface and cave populations consisted of all age groups, but cavefish in the 4^+ and 5^+ age groups had larger average standard lengths than the surface fish. Furthermore, the largest *T. karsticus* individual ever recorded (150 mm standard length) was caught in the cave during this study. Conversely, fish in the 2^+ age group were larger in the surface population (Marčić et al. in preparation). Wild-caught cavefish also had a higher liver fat content (and whole-body content, although not statistically significant; Supplementary Figure S2) and showed changed expression in many genes related to the metabolism of lipids, carbohydrates, and amino acids.

Most studied traits in the wild *T. karsticus* cavefish exhibited adaptive changes resembling the phenotypes observed in obligate cave-dwellers. However, compared to the surface fish, the cavefish displayed thicker retinal layers, except for a thinner retinal pigment epithelium, possibly reflecting reduced melanin pigmentation, as observed in the skin. Interestingly, the inner nuclear layer, outer nuclear layer, and photoreceptor cell layer also show significant thickening in dark-raised *Astyanax* surface fish (Bilandžija et al., 2020). Given that eye reduction and loss are hallmarks of adaptation to subterranean environments, the plasticity of retinal morphology may be considered maladaptive, aligning with the random nature of plastic responses in adaptive outcomes (Ghalambor et al., 2007). The similarity of these results in both *Astyanax* and *Telestes* species suggests negative selection against initial maladaptive plasticity may play a role in subsequent evolution of eye regression.

The cavefish exhibited higher variance in all investigated phenotypic traits compared to surface fish, consistent with the expectation of increased phenotypic variation in novel or extreme environments (Radersma et al., 2020). This higher variance suggests the release of hidden genetic variation in response to stress and exposure to novel and challenging environments, potentially explaining why cavefish occupy a much larger phenotypic space than surface fish. Previous research on *A. mexicanus* demonstrated that exposure to low conductivity water can result in higher variance in eye size in fish due to heat shock protein 90 (HSP90)-mediated release of hidden genetic variation (Rohner et al., 2013). Standing genetic variation has been implicated in the colonization of novel habitats in other systems, as well as in independent lineages of cave-adapted *Asellus aquaticus* (Barrett & Schluter, 2008; Kingman et al., 2021; Rodas et al., 2023). However, phenotypes of some of the cavefish still fell within the range exhibited by surface fish. One possibility is that they were swept into the cave recently, with insufficient time to develop plastic responses. Alternatively, these individuals may simply be less plastic. If so, this would indicate that the surface population is large enough and sufficiently diverse to contain necessary standing genetic variation for plasticity.

To test the involvement and extent of plasticity associated with cave colonization in *T. karsticus*, we exposed fish to complete darkness (DD) or light/dark (LD) photic cycle. The morphometric differences between wild cavefish and surface fish largely disappeared after 7 months, and completely disappeared after 22 months, with no difference in any morphometric trait between the two morphs. Furthermore, within each morph, all morphometric traits changed in response to laboratory husbandry (with only a few exceptions in cavefish). These results suggest that the morphometric traits studied are plastic and that the differences in shape and size of the external habitus between the surface fish and cavefish are due to phenotypic plasticity. In addition, we found no evidence of genetic differentiation between the two morphs, with all genetic variance attributed to differences among and not within populations. Although our results suffer from small sampling size, they provide further evidence that some phenotypes in the cave population of *T. karsticus* are the result of phenotypic plasticity and not local genetic adaptation.

There were no evident changes in most traits between the LD- and DD-maintained fish of the same morph. One possible explanation may be the small sample size at the end of the experiment. A malfunction in the automatic feeder caused the death of more than half of the experimental fish in a single weekend. Furthermore, as the number of fish that could be taken from nature was limited by the permit and the incident occurred in the late stages of the experiment, we did not replenish the laboratory stock. Another possibility may be that our laboratory conditions were too homogeneous, and therefore, the phenotypic differences observed between wild surface fish and cavefish may be influenced by environmental factors other than just complete darkness. Sušik cave is likely to exhibit variations in food availability and quality, microclimate conditions, fluctuations in water chemistry, and distinct biotic interactions, including the presence of predators or parasites as well as intra- and interspecific competition, all of which may contribute to the phenotypic differences observed in natural populations. Nevertheless, we observed similar trait changes, such as lower pigmentation and higher fat levels, in the dark-maintained surface fish, suggesting that these traits may have arisen due to the exposure of surface fish to a cave-like environment. These trait changes were triggered even though the fish were exposed to experimental conditions as adults, indicating that phenotypic changes can occur after the completion of development, the most sensitive period for perturbation, and that adult homeostatic responses, sometimes referred to as acclimatization or acclimation, can also contribute to the development of specialized cave traits. In addition, gene expression patterns observed in surface fish kept in constant darkness showed similarities to those observed in cavefish compared to surface fish in the wild, suggesting that gene expression patterns in cave populations may be influenced by plastic responses in surface fish colonizers.

In contrast to surface fish, we found no significant differences in any studied phenotype between the experimental laboratory groups of cavefish, and few significant differences between cavefish in the wild and laboratory conditions. In addition, at the gene expression level, the laboratory cavefish groups exhibited fewer DEGs, and their expression patterns were in the opposite direction compared to both wild and surface fish comparisons, with unique GO term signatures. These results suggest that cavefish may

have evolved lower levels of plasticity, similar to *A. mexicanus* cavefish (Bilandžija et al., 2020). Considering the potentially young evolutionary age of the *T. karsticus* cavefish population and the fact that they do not display a fully adapted cave phenotype, this finding is surprising. In a stable environment under permanent darkness, any plasticity that incurs costs would be selected against. If there is no environmental variation driving selection for plasticity, neutral processes and drift may also lead to the erosion of plasticity (Hall & Colegrave, 2008; Murren et al., 2015). This could occur if cavefish are unable to migrate back to the surface and are therefore never re-exposed to the LD photic cycle. However, if that is the case, it raises the question of how surface fish retain plasticity in response to darkness. Populations can exhibit adaptive plastic responses to novel environments if they or their ancestors have been previously exposed to such conditions (Bonamour et al., 2019; Fox et al., 2019). One possibility is the occurrence of rare and massive floods, occurring once in a decade, century, or millennium but within evolutionarily relevant timescales. These floods have the potential to cover extensive areas of the Dinaric Karst, allowing for the mixing of surface and cave populations (Trontelj, 2022) and the exchange of alleles that mediate plasticity to darkness. Such a scenario has been proposed to explain the distribution and migration patterns of certain Dinaric Karst cave-dwellers, which occupy large ranges and hydrologically isolated river basins without apparent genetic differentiation (Zakšek et al., 2009). Of note, in addition to phenotypes, plasticity is also likely to evolve in response to darkness during cave colonization. However, it is also possible that the observed differences in plasticity between cave and surface populations may be due to inherent differences in their ability to adjust to our laboratory conditions. Cave and surface environments differ in many features other than darkness, which are not easily replicated in a laboratory setting. Exploring these additional features would contribute to a more comprehensive understanding of the evolution of plasticity in *T. karsticus*.

In conclusion, our study on *T. karsticus* demonstrated the value of this species as a model system for investigating the processes of cave colonization and the evolution of specialized cave traits in the presence of gene flow from the surface. Notably, *T. karsticus* maintained a year-round presence in Sušik cave and exhibited the ability to reproduce underground, making it a unique species in the Dinaric Karst region. Our findings revealed distinct phenotypic differences between the cave and surface populations in the wild, including smaller eyes, reduced pigmentation, increased weight and body width, and enhanced fat deposition. Many of these differences are similar to those present in obligate cave-dwellers as adaptations to subterranean environments. In the common garden experiment, surface fish exposed to complete darkness, an ecologically relevant trait common to all subsurface habitats, exhibited several trait changes similar to wild cavefish, including reduced pigmentation and increased fat deposition. However, the maintenance in common laboratory conditions induced many significant changes in morphometric traits within each fish population and erased all differences between the two morphs. These results suggest that traits related to body shape and size are plastic and that phenotypic plasticity may have been the driver of these trait changes in cavefish in the wild. Our findings also suggested that alongside developmental plasticity, adult homeostatic responses, such as acclimatization to the cave environment, may have played a significant role in the emergence of specialized cave traits.

SCIENTIFIC FIELD SURVEY PERMISSION INFORMATION

Permit no. UP/I-612-07/20/48/84; 517-05-1-1-20-3 issued by the Republic of Croatia Ministry of Environmental Protection and Energy.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Concept and design: H.B.; acquisition of data: M.Č., Z.M., M.L., R.G., and H.B.; analysis and interpretation of data: M.Č., Z.M., M.L., R.G., and H.B.; first draft of the article: M.Č. and H.B.; critical revision of intellectual content: M.Č., Z.M., M.L., R.G., and H.B.; final approval of the article: M.Č., M.L., Z.M., R.G., and H.B. All authors read and approved the final version of the manuscript.

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