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Transcriptomic Insights into the Loss of Vision in Molnár János Cave's Crustaceans

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Synopsis Animals that inhabit subterranean environments often undergo various distinct phenotypic modifications (referred to as "troglomorphy") as they transition to life in perpetual darkness. However, the molecular basis behind troglomorphy remains poorly understood, particularly in regards to the mechanisms involved in the reduction and/or loss of traits at the transcriptomic level. In this study, we investigate the transcriptional basis behind vision loss in populations of cave-dwelling crustaceans. We employ phylogenetic and transcriptomic methods on surface and cave-adapted populations of an emerging model species for biospeleology, the isopod *Asellus aquaticus* (Linnaeus, 1758), and the amphipod *Niphargus hrabei* S. Karaman, 1932. These two species show contrasting directionality in the surface-cave transition, which positions them as ideal study subjects. *Asellus aquaticus* is common in surface waters and is only occasionally found in caves, where its populations present different degrees of eye reduction and pigmentation. On the other hand, the eyeless *N. hrabei* has successfully colonized surface environments despite belonging to an almost exclusively cave-dwelling genus. By sequencing and assembling robust *de novo* transcriptomes we characterized differences in visual genes and pathways among surface and cave populations of the aforementioned species. Our results indicate that despite having reduced eyes, recent cave colonizer *A. aquaticus* is still capable of expressing functional visual opsins and major components of the phototransduction pathway within the cave. *Niphargus hrabei*, a species with an ancient cave origin, shows no clear indication of being capable of sight. However, the expression of putative functional visual opsins and other phototransduction genes was maintained, which suggests that this eyeless species might be capable of extraocular photoreception. With the present study, we aim to bring forth the Molnár János Cave system as a promising research avenue to improve our understanding of patterns of reduction and loss of vision in caves and other aphotic environments.

Introduction

As populations of animal species colonize and adapt to life in the perpetual darkness of caves, they are often subject to a suite of morphological, physiological, and behavioral changes that are collectively described as "troglomorphic" (Porter and Crandall 2003; Mejía-Ortíz et al. 2006; Pérez-Moreno et al. 2016). Cave-dwelling animals typically exhibit dichotomous troglomorphic traits, classified as either progressive/constructive or regressive/reductive depending on the assumed directionality of these changes (Porter and Crandall 2003; Mejía-Ortíz et al. 2006; Pérez-Moreno et al. 2016). Examples of progressive troglomorphic changes include improved spatial orientation and olfaction as well as larger sensory and ambulatory appendages (Turk et al. 1996; Li and Cooper 2001, 2002; Mejía-Ortíz and Hartnoll 2006). By contrast, regressive troglomorphic modifications typically feature a decrease or loss of characters in comparison with their surface-inhabiting counterparts (Pérez-Moreno et al. 2016). Among these regressive traits one may find those that are more commonly associated with subterranean life such as slower metabolism, little or no pigmentation, and the reduction or loss of vision and visual structures (Sket 1985; Wilkens 1986; Mejía-Ortíz and López-Mejía 2005; Bishop and Iliffe 2009).

Troglomorphic phenotypes, with reduced or absent visual structures, have arisen repeatedly and independently across the phylogenetic breadth of the animal kingdom in response to analogous environmental conditions (Caccone and Sbordoni 2001; Protas et al. 2007; Pérez-Moreno et al. 2016). To this day, most comprehensive studies of cave fauna's regressive evolution have focused on freshwater fish, particularly the Mexican Cave Tetra *Astyanax mexicanus* (Porter and Crandall 2003). This species has served an important role as a model organism for evolutionary and developmental biology, facilitated by the presence of "normal" surface populations, numerous independent cave colonization events, and varying degrees of eye loss (Jeffery and Martasian 1998; Yamamoto and Jeffery 2000; Jeffery 2001). This has led to significant insights regarding the diverse mechanisms by which eyes and vision can be lost in vertebrates. In comparison, invertebrates have received little attention, despite often comprising the majority of the animal diversity found in caves (Protas et al. 2011; Pérez-Moreno et al. 2016). Given the global diversity of cave invertebrates and phenotypic convergence that has arisen from a multitude of independent colonization events; these organisms can be considered ideal subjects to study the predictability of evolution, particularly in regards to the loss of complex traits such as vision (Pérez-Moreno et al. 2016).

Recent advances in cave diving technology and in the field of genomics now offer an unprecedented opportunity to investigate the molecular basis and evolution of vision loss in subterranean organisms (Pérez-Moreno et al. 2016). Likewise, molecular techniques have been pivotal for increasing our understanding of invertebrate vision and the genes and pathways underlying this trait (Cronin and Porter 2014). However, to date there are relatively few empirical studies of loss of function in visual genes and pathways in subterranean environments (Niemiller et al. 2013). This absence is especially noted for those studies that incorporate high-throughput sequencing techniques (Pérez-Moreno et al. 2016). Genomic and transcriptomic methods, such as RNA sequencing, have proven useful tools to examine the pathways that determine an organism's ability to visually detect light (i.e., the phototransduction pathway; Porter et al. 2012). The phototransduction pathway is activated through the detection of specific wavelengths of light by visual pigments composed of an opsin protein bound to a chromophore (e.g., 11-*cis* retinal; Nathans 1987; Tierney et al. 2015). Opsins are a diverse group of photoreceptor proteins found in metazoans that are essential for animal vision (Terakita 2005). Belonging to the G-protein-coupled receptors family (GPCRs), these membrane-associated proteins play important roles in both visual and non-visual phototransduction (Shichida and Matsuyama 2009). When bound to a chromophore, opsins form photon-absorbing pigments that activate G-proteins when exposed to light (Nathans 1987). This exposure thus initiates the phototransduction signaling cascade, which is tightly linked to regulation of circadian rhythms, phototactic behaviors, and most importantly, vision (e.g., Arendt et al. 2004; Shichida and Matsuyama 2009; Pérez-Moreno et al. 2018). Among arthropods, the phototransduction pathway has been well characterized in *Drosophila* (review, Hardie and Juusola 2015) and significant progress has been made in the xiphosuran *Limulus polyphemus* (review, Battelle 2017), but efforts to do so in the speciose sub-phylum Crustacea have only started to gain momentum (review, Cronin and Porter 2014; Porter 2016; Porter et al. 2017).

The isopod *Asellus aquaticus* (Linnaeus, 1758) and the amphipod *Niphargus hrabei* S. Karaman, 1932 are two crustacean species that function as ideal and complementary systems to explore questions regarding the loss of eyes and vision. *Asellus aquaticus* is a widespread species of freshwater isopod found in surface waters throughout Europe that occasionally colonizes caves. In many known cave populations, *A. aquaticus* is found with distinct troglomorphic morphotypes with varying degrees of eye reduction (Sket 1994; Protas et al. 2011; Konec et al. 2015; Stahl et al. 2015; Pérez-Moreno et al. 2017; Fig. 1). Only a small number of these subterranean populations are known to be completely blind (e.g., in Slovenia and Romania, Sket 1994), as the visual status of most populations has not been evaluated. For example, Hungary's Molnar Janos Cave is inhabited by a troglomorphic and depigmented *A. aquaticus* population with reduced eyes (Pérez-Moreno et al. 2017) whose visual status remains unknown. Unlike their blind cave conspecifics elsewhere, this population's reduced eyes have both pigmentation and ommatidia. *Niphargus hrabei*, on the other hand, is an almost exclusively cave-dwelling genus that is hypothesized to have ancient cave-origins and lacks eyes in both surface and cave populations (Balázs et al. 2015; Copilas-Ciocianu et al. 2017; Pérez-Moreno et al. 2017; Fig. 1). At first glance *N. hrabei* appears to have an "eyespot" with yellow pigmentation proximal to the antennal peduncle as its congener *N. valachicus* does, which upon closer examination has not shown any identifiable histological structure (C. Fišer, personal communication). Furthermore, these two species, which are distributed

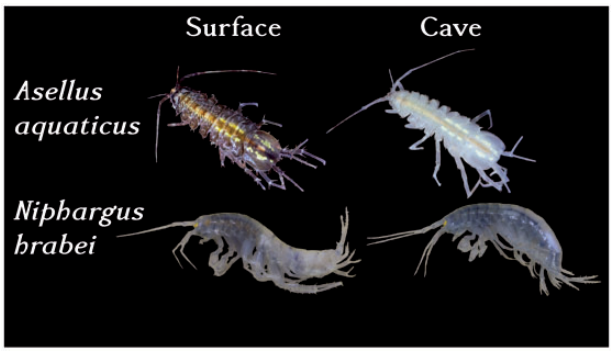

Fig. 1 *Asellus aquaticus* is troglomorphic only within the cave environment, while *Niphargus hrabei*'s morphotype remains constant across environments (adapted from Pérez-Moreno et al. 2017).

across a wide-area of Central and Eastern Europe, are sympatric above and below ground.

The present study aims to investigate the molecular basis behind the loss of vision, with an emphasis on the phototransduction pathway, via transcriptomic and phylogenetic approaches. Specifically, we aimed to characterize visual opsins in surface and cave populations, as well as assessing the presence of other phototransduction genes that are indicative of photoreception. This investigation was undertaken by capitalizing on natural experimental conditions generated by the presence of *A. aquaticus* and *N. hrabei* in the Molnár János Cave system (Budapest, Hungary). Despite having markedly different selective pressures, Molnár János Cave, the adjacent Malom Lake, and the Danube River are all inhabited by both *A. aquaticus* and *N. hrabei* (Pérez-Moreno et al. 2017). As such, this system facilitates comprehensive and intricate investigations regarding the adaptation and evolution of cave fauna.

Methods

Specimen collection

Specimens of *A. aquaticus* and *N. hrabei* were sampled from Rakos Rock in Molnár János Cave (Fig. 2) using a "Sket bottle" (Chevaldonné et al. 2008) following the methods of Pérez-Moreno et al. (2017). Cave specimens were preserved *in situ* immediately upon resurfacing from the dive (within the subterranean tunnel connecting to the Kessler Hubert air chamber) to minimize stress to the animals and any possible transcriptional response activity resulting from exposure to light and/or light-induced photoreceptor damage (Meyer-Rochow 2001). Surface specimens of both species were additionally sampled from surface populations (Malom Lake and the Soroksár branch of the Danube River; Fig. 2). As with the cave specimens, surface individuals were also preserved *in situ* and immediately upon collection. Following the aforementioned preservation in RNAlater® (ThermoFisher Scientific), the samples were frozen at −80°C to prevent nucleic acid degradation and thus allow for subsequent RNA isolation, sequencing, and transcriptomic analyses.

Data and quality control

Total RNA was individually extracted from two whole specimens per species (*A. aquaticus* and *N. hrabei*) per population (Molnár János Cave, Malom Lake, Soroksár) using TRIzol® reagent (ThermoFisher Scientific). Each specimen's RNA was prepared for sequencing in individual reactions with an initial rDNase® (Macherey-Nagel®) treatment, and subsequent mRNA isolation, cDNA synthesis, and barcode/adapter ligation with a NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina®. The resulting barcoded libraries were size-selected with a Pippin Prep® instrument (Sage Science), pooled in equimolar concentrations, and sent for sequencing on an Illumina® HiSeq 4000 lane at GENEWIZ, Inc.

Quality of the raw sequencing reads was evaluated via FastQC (Andrews 2010) prior to the assembly process to inform quality and adaptor trimming with Trimmomatic 0.36 (Bolger et al. 2014). Trimmed and quality-filtered reads were then piped to *k*-mer based Rcorrector (Song and Florea 2015) for correction of random sequencing errors and to BBNorm for read depth normalization prior to assembly.

Transcriptome assembly and post-processing

The clean and normalized sequencing reads were assembled into *de novo* transcriptomes for each population and species with the Trinity pipeline (version 2.5.0; Grabherr et al. 2011; Haas et al. 2013), specifying a minimum contig length of 200 bp, a *k*-mer size of 23, and only retaining transcripts supported by read mappings. Post-processing of the *de novo* assemblies consisted of removal of duplicate transcripts and rRNA sequences (dedupe.sh and BBDuk from the BBTools suite, available at: http://sourceforge.net/projects/bbmap). Transrate 1.0.3 and Benchmarking Universal Single-Copy Orthologs (BUSCO) 3.0.2 (Simão et al. 2015; Smith-Unna et al. 2016) were then, respectively, employed to calculate summary statistics and to assess the completeness of the transcriptome assemblies. BUSCO evaluates transcriptome completeness in an evolutionary informed context by evaluating the presence and/or fragmentation of universal single copy

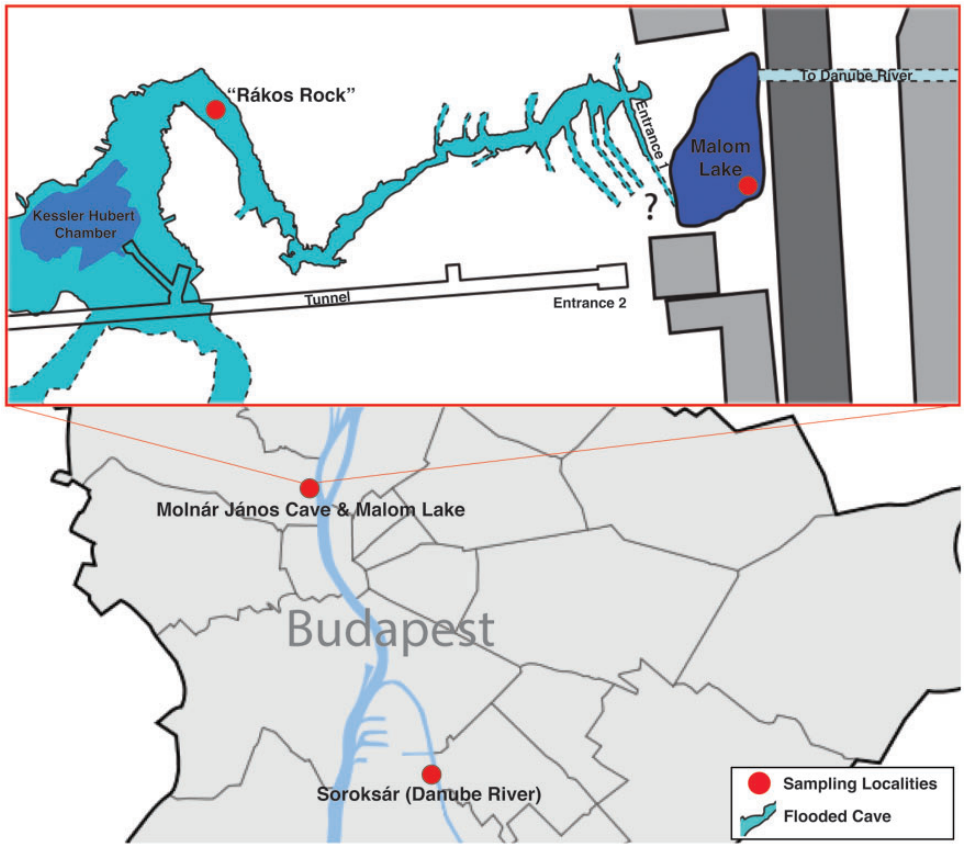

Fig. 2 Specimens of both species were sampled from three localities within Budapest, Hungary. Circles indicate exact sites within Molnár János Cave (Rákos Rock) and surface environments (Malom Lake and Soroksár [Danube River]; adapted from Pérez-Moreno et al. 2017).

orthologs (Simão et al. 2015). BUSCO analyses were conducted using OrthoDB's Arthropoda database of orthologous groups ($n = 1066$; Waterhouse et al. 2013) as a reference dataset.

Phylogenetic identification and annotation of visual genes

A suite of phylogenetic tools were employed to identify genes involved in the phototransduction pathway and functionally classify putative opsins in our *de novo* transcriptomes, as part of a previously described pipeline (Pérez-Moreno et al. 2018). In essence, this pipeline extracts all the open-reading frames (ORFs) found within the transcriptome and then runs these data through a modified command-line version of the phylogenetically-informed annotation (PIA) tool (Speiser et al. 2014). Briefly, PIA allows for the identification of visual genes by initial BLAST searches against a database of known visual genes, and subsequent alignment and placement of BLAST hits in pre-computed phylogenies to differentiate false-positives from the genes of interest. The modified pipeline extends PIA's functionality by integrating additional steps of unbiased automated pruning of tree branches exceeding a pre-specified threshold (in this case, 4× mean absolute deviation of branch lengths) to remove false-positive hits, and redundancy reduction, which results in more concise and cleaner candidate gene lists (for more details see Pérez-Moreno et al. 2018).

Finally, opsins were further investigated as they are initiators and essential components of the phototransduction cascade. The specific amino acid residues that interact with the chromophore to form a functional photopigment have a direct influence on the opsin's spectral sensitivity by permitting the absorption of distinct wavelengths of light (Imai et al. 1997; Kuwayama et al. 2002; Porter et al. 2007; Katti et al. 2010). This direct correlation between opsins' amino acid compositions with specific spectral sensitivities not only allows for fine scale spectral tuning at both ecological and evolutionary timescales, but also makes opsins particularly suitable to functional classification by sequence similarity and phylogenetic approaches (Mirzadegan et al. 2003; Wong et al. 2015; Matsumoto and Ishibashi 2016; Pérez-Moreno et al. 2018). As such, the previously

identified putative opsins were aligned with PROMALS3D (Pei and Grishin 2014) using a large opsin reference dataset ($n = 910$; Porter et al. 2012) that includes the main types of opsin subfamilies and representatives of a variety of spectral sensitivities from an extensive taxonomic range. A final opsin phylogenetic tree was then reconstructed with IQ-tree (Nguyen et al. 2015), a maximum-likelihood based phylogenetics software package that has performed favorably in recent benchmarks (Zhou et al. 2018). Phylogenetic reconstruction was undertaken using an LG general amino acid replacement matrix, under a FreeRate model with nine rate categories, and empirical base frequencies (LG+R9+F; Le and Gascuel 2008; Soubrier et al. 2012) as suggested by ModelFinder (Kalyaanamoorthy et al. 2017). Subsequently, split support was assessed using three different methodologies: an Ultra-fast bootstrap approximation (UFBoot; 10,000 replicates), a Shimodaira–Hasegawa-like approximate likelihood ratio test (SH-aLRT; 10,000 replicates), and an approximate Bayes test (Guindon et al. 2010; Anisimova et al. 2011; Minh et al. 2013).

Results and discussion

The present study aimed to identify and characterize phototransduction genes, with an emphasis on visual opsins, to enable intra- and inter-specific comparisons among surface and cave populations with varying eye phenotypes of *A. aquaticus* and *N. hrabei*. For this purpose, we have contributed additional cave and surface *A. aquaticus* transcriptomes from previously unstudied populations, in addition to the first transcriptomic resources available for the speciose and wide-spread genus *Niphargus*. Here, we present and discuss this study's *de novo* transcriptome assemblies, their summary statistics, and completeness. Secondly, we compare and contrast the cave and surface transcriptomes in search of visual genes and those involved in the phototransduction pathway. In light of our results, we infer the functional status of their visual opsins and discuss the future prospects for this emerging study system.

Transcriptome sequencing and assembly

Over 380 M reads and approximately 115 gigabases of data were obtained for our 12 samples, respectively, yielding approximately 32 M reads and 9.5 gigabases per individual. Raw sequencing data are available on the NCBI's Sequence Read Archive database under Bioproject PRJNA476149 and accession numbers for identified genes are provided in the Supplementary Materials. The quality of our *de novo* transcriptome assemblies was evaluated by examining the summary statistics as reported by Transrate (Smith-Unna et al. 2016) and the results of the BUSCO (Simão et al. 2015) assessment of completeness. *Asellus aquaticus* transcriptome assemblies recovered 86903–123735 contigs with a mean sequence length of 938–1076 base pairs (Table 1). Of these, 25562–30264 sequences contained ORFs designating them as putative protein-coding genes. The notably different transcriptome sizes in this species could be indicative of the different pathways involved with living in such contrasting environments, which is also reflected by the distinct phenotypes found in them. Transcriptome contiguity was evaluated by calculating N50 values, a commonly employed metric to assess the contiguity of a *de novo* transcriptome assembly. *Asellus aquaticus de novo* transcriptomes had N50 values ranging from 1737 bp to 2022 bp, which are more than twice as long as the only previous transcriptomic study for this species (Stahl et al. 2015). Similarly, the *de novo* transcriptomes for *N. hrabei* were comprised of 107451–134592 contigs with a mean sequence length of 874–883 bp. The N50 values for *N. hrabei* transcriptomes range from 1598 bp to 1648 bp, which also compare favorably with previous amphipod transcriptome studies (e.g., Lan et al. 2017). Additional metrics for our *de novo* transcriptomes are given in Table 1.

Transcriptome completeness of our *de novo* transcriptomes, as assessed by BUSCO, was favorable for both species (Table 2). In *A. aquaticus* we were able to find 90.1–93.6% complete sequences of the 1066 genes employed for benchmarking in Arthropoda. An additional 3.3–6.2% were present but fragmented, and only 2.7–3.9% were not found in the transcriptome. Similarly, *N. hrabei*'s transcriptome was found to be nearly complete but slightly more fragmented with 82–90.5% full-length BUSCO genes, 5.3–7.9% fragmented, and 4.2–10.5% missing. *Niphargus hrabei*'s larger proportion of missing BUSCOs, despite equivalent sequencing depth, might reflect ancestral gene-loss patterns due to reductive troglomorphy. Future explorations of speleotranscriptome completeness in Niphargidae and other ancient stygobiont/troglobiont lineages are needed to test this hypothesis.

Transcription of opsins and the phototransduction pathway

Phylogenetic analyses revealed an almost complete phototransduction pathway, as defined in the PIA tool (Speiser et al. 2014), for the transcriptomes of

Table 1 Summary statistics for the *de novo* transcriptome assemblies produced as part of this study

	Asellus aquaticus			*Niphargus hrabei*		
Metric	**Molnár János Cave**	**Malom Lake**	**Soroksár (Danube River)**	**Molnár János Cave**	**Malom Lake**	**Soroksár (Danube River)**
Number of sequences/contigs	123,735	86,903	98,343	112,658	107,451	134,592
Longest sequence/contig (bp)	26,685	23,685	29,698	16,763	13,022	24,002
Number of bases	125,035,326	93,533,167	92,258,922	98,447,524	94,883,864	118,581,253
Mean transcript/contig length (bp)	1011	1076	938	874	883	881
Number of transcripts/contigs >1000 bp long	34,381	27,588	26,257	27,492	27,582	32,435
Number of transcripts/contigs >10,000 bp long	272	122	117	66	17	157
Number of transcripts with ORFs	30,264	25,562	26,989	20,669	21,547	27,159
Mean ORF percent	50.42	53.29	55.98	46.08	46.32	48.53
N50	2003	2022	1737	1624	1598	1648
N30	3410	3280	2932	2776	2669	2870
N10	6398	5868	5552	5180	4699	5626
GC content	0.35	0.36	0.37	0.41	0.41	0.42

Table 2 Results of transcriptome completeness assessment by Benchmarking Universal Single-Copy Orthologs using OrthoDB's Arthropoda database of orthologous genes

Species	**Transcriptome**	**Complete BUSCOs (%)**	**Fragmented BUSCOs (%)**	**Missing BUSCOs (%)**	**Total BUSCOs searched**
Asellus aquaticus	Molnár János Cave	93.6	3.7	2.7	1066
	Malom Lake	92.8	3.3	3.9	
	Soroksár (Danube River)	90.1	6.2	3.7	
Niphargus hrabei	Molnár János Cave	82	7.5	10.5	
	Malom Lake	84.1	7.9	8.9	
	Soroksár (Danube River)	90.5	5.3	4.2	

A. aquaticus (Table 3), which suggests that the cave population might retain the ability to detect light despite their reduced eyes. Genes identified included major components of the phototransduction pathway, i.e., initiators of the cascade (opsins), regulatory components (Gq proteins, trp ion channels), and signal terminators such as arrestin and the retinal degeneration (rdg) genes. Studies are ongoing to determine if these genes are potentially down-regulated in the cave versus the surface populations or if alternate mechanisms are at play. It is possible that the expression of phototransduction genes could be maintained at baseline levels, while vision loss is occurring by other means. For example, previous studies have shown a reduction or loss of optical ganglia and respective brain regions in other cave-adapted crustaceans (Stegner et al. 2015). *Niphargus hrabei* cave transcriptome contained fewer phototransduction genes (Table 3), which could be attributed to the cave origin of this ancient genus (circa 88 mya; McInerney et al. 2014) leading to pseudogenization events. It is important to note that the absence of a gene in a transcriptome does not necessarily indicate that such gene has been pseudogenized or lost from the genome (where it may in fact remain in a functional form), but rather than it is simply not being expressed in the given conditions. However, the expression of genes associated with rhabdomeric photoreception (trp and rdgB) in both photic environments suggests that, despite *N. hrabei*'s lack of eyes and therefore blindness, the possibility of extraocular photoreception in this species cannot be discarded.

Following identification and annotation of putative opsins, these were aligned with a reference dataset for a final step of phylogenetic functional annotation. As a result, a large phylogeny (Fig. 3) was produced where putative opsins were assigned functional identities and spectral sensitivities were inferred based on their phylogenetic position.

Table 3 Phototransduction pathway genes identified by PIA (Speiser et al. 2014) in the cave and surface transcriptomes of *A. aquaticus* and *N. hrabei*

	Asellus aquaticus			*Niphargus hrabei*		
Gene	Molnár János Cave	Malom Lake (Surface)	Soroksár (Surface)	Molnár János Cave	Malom Lake (Surface)	Soroksár (Surface)
Arr	•	•	•			
DAGK	•	•	•	•	•	•
GPRK1	•	•	•	•	•	•
GPRK2	•	•	•		•	•
Gq_alpha	•	•	•	•	•	•
Gq_beta	•	•	•	•	•	•
Gq_gamma						
r_opsin	•	•	•	•	•	•
PKC						•
PLC	•	•	•	•	•	•
rdgB		•	•		•	•
rdgC	•					
trp	•	•	•			•

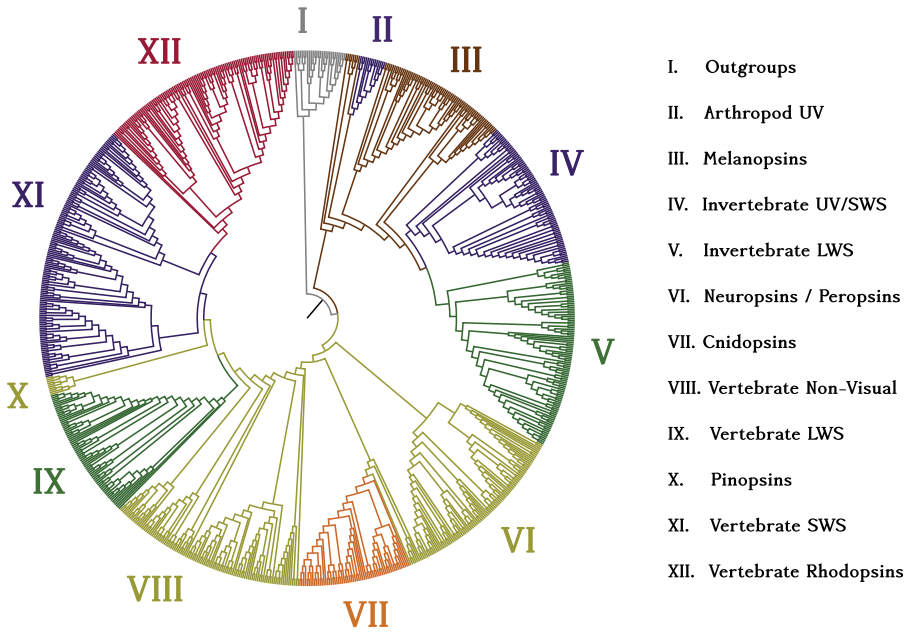

Fig. 3 Circular phylogeny of identified and reference opsins illustrating major clades (denoted in roman numerals) that correspond to their functional classification.

Asellus aquaticus transcriptomes contained a single short wavelength sensitive/UV sensitive opsin and one long wavelength sensitive opsin, which did not differ significantly between surface and cave populations (Fig. 4). *Niphargus hrabei* on the other hand was found to express no SWS/UV opsins, but a single LWS opsin that was also identical in all populations (surface and cave populations, Fig. 4). Pairwise sequence alignments of each species and morphotype's opsins were compared with reference opsin sequences, bovine (*Bos taurus*) and squid (*Todarodes pacificus*) rhodopsins (Accessions NP_001014890 and CAA49906, respectively) to further elucidate any amino acid substitutions differences among populations and their possible effects on function and inferred spectral sensitivities (Table 4; Palczewski et al. 2000; Takahashi and Ebrey 2003; Katti et al. 2010).

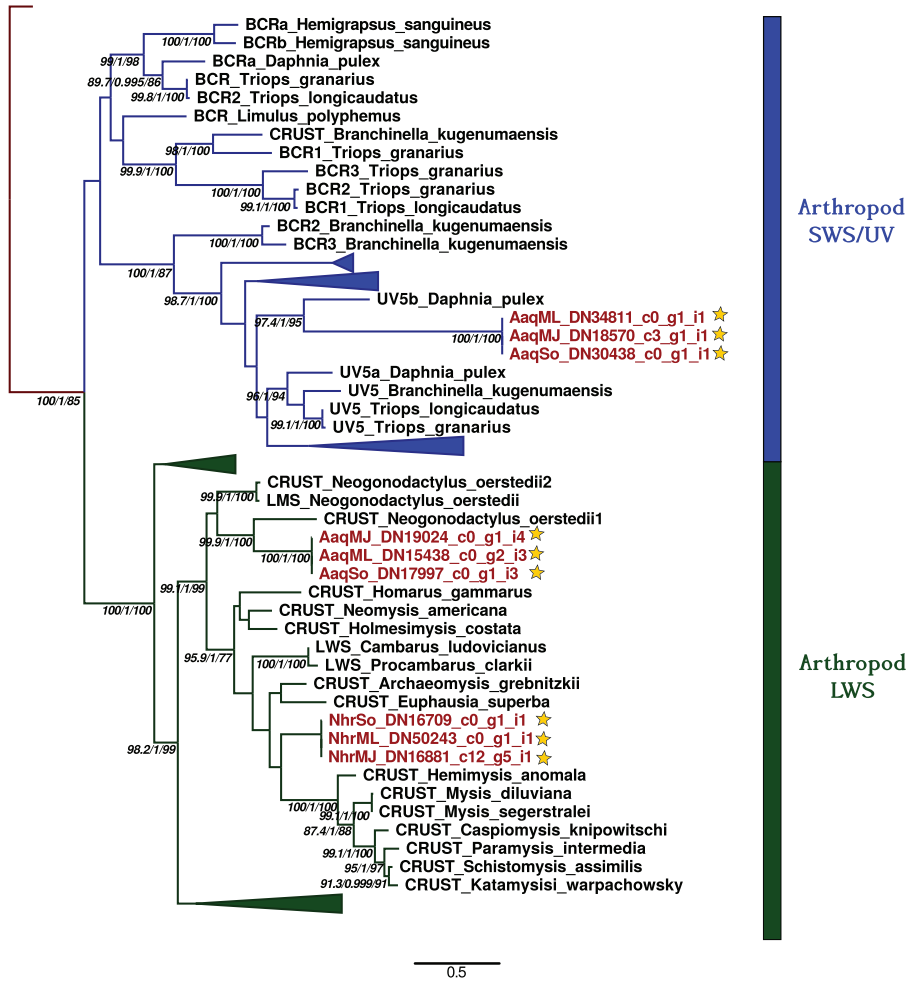

Fig. 4 Expanded view of the Arthropod LWS and Arthropod SWS/UV clades. Non-crustacean clades were collapsed for conciseness. Support values correspond to SH-aLRT/aBayes/UFBoot and are not shown when bootstrap support <75. Both *A. aquaticus* and *N. hrabei* express functional LWS opsins low intraspecific differentiation. All populations of *A. aquaticus* expressed the same SWS/UV opsin regardless of their morphotype. In *Niphargus hrabei* transcriptomes, SWS/UV opsins were not found.

All of the putative opsins identified by our analyses, including those from cave populations, appear to be functional based on the presence of the functionally conserved residues of opsins and other GPCRs (Arakawa et al. 2011) involved in Schiff base linkage (Lys296), disulfide bond formation (Cys110 and Glu113), stabilization (Cys138), and signal propagation (Glu134–Arg135–Tyr136 motif; Li et al. 2004; Arakawa et al. 2011). The opsin annotated as SWS/UV for *A. aquaticus* contains a lysine residue in the site corresponding to position 90 in bovine rhodopsin, further supporting its putative UV sensitivity as inferred from the phylogeny (Table 4; Salcedo et al. 2003). It is thus plausible that the expression of this opsin has not been fully lost within the cave, despite the lack of light, due to the recent divergence (~140 kya; Pérez-Moreno et al. 2018) of *A. aquaticus* Molnár János Cave and nearby surface populations. On the other hand, both *A. aquaticus* and *N. hrabei* have LWS opsins that only differ between the two species at bovine rhodopsin site 126 and 269, the significance of which yet remains to be investigated. Additionally, LWS opsins for both species show a serine residue at site 292 instead of an alanine, which has been previously shown to be able to shift invertebrate spectral sensitivity in either direction (Salcedo et al. 2009; Katti et al. 2010).

Asellus aquaticus transcriptome analyses show that major key components of the phototransduction pathway are expressed in all populations investigated. Cave *A. aquaticus* express the same opsin genes as surface individuals (Fig. 4), with only minor differences. The SWS/UV opsin found in *A. aquaticus* is identical in all populations except for their total length. However, there is no other indication that they consist of different isoforms, which

Table 4 Amino acids present in sites known to influence spectral sensitivity for each identified opsin

Species and opsin type	Population	Bovine G^{90} SquidG	Bovine E^{113} SquidY	Bovine T^{118} SquidG	Bovine E^{122} SquidF	Bovine W^{126} SquidM	Bovine A^{164} SquidS	Bovine H^{211} SquidG	Bovine A^{269} SquidA	Bovine A^{292} SquidV	Bovine A^{295} SquidA
Asellus aquaticus SWS/UV	Molnár János Cave Malom Lake (surface) Soroksár (surface)	K	Y	A	P	V	P	V	A	A	C
Asellus aquaticus LWS	Molnár János Cave Malom Lake (surface) Soroksár (surface)	M	Y	S	C	Y	S	V	C	S	A
Niphargus hrabei LWS	Molnár János Cave Malom Lake (surface) Soroksár (surface)	M	Y	S	C	W	S	Y	L	S	A

Notes: Position numbers depicted refer to the equivalent sites in the reference rhodopsin sequences (the bovine *Bos taurus* and the squid *Todarodes pacificus*).

suggests that the length is due to fragmentation during assembly. *Asellus aquaticus* LWS opsin in Malom Lake shows an aspartic acid to glutamic acid substitution at position 12, which suggests that the mutation is inconsequential given its extracellular location (proximal to the N-terminus) and the physicochemical similarities of said residues. A similar occurrence is observed in *N. hrabei*, where the Soroksár (Danube River) surface population shows a single serine to arginine substitution. There were no indications of pseudogenization in neither opsins nor other expressed phototransduction genes, as has been the case in other subterranean animals (Yokoyama et al. 1995; Kim et al. 2011; Hinaux et al. 2013; Rétaux and Casane 2013). The lack of significant differentiation between the cave and surface population's expressed opsins is perhaps not that surprising, given recent divergence-time estimates based on molecular clocks (circa 139,000 years ago for *A. aquaticus*; Pérez-Moreno et al. 2017). An *A. aquaticus* study by Protas et al. (2011) found a candidate gene (*lim1*) that appeared to be linked with loss of eyes, by employing reduced-representation DNA sequencing techniques and QTL analyses. However, this gene was not thought to be responsible for the loss of this trait, but rather a gene located in its genomic vicinity (Protas et al. 2011). Future research with the use of whole genome sequencing will undoubtedly help determine if drift or selective forces are acting in areas upstream of the opsin gene, leading to reduced but detectable transcription.

Subterranean diving beetles from Australia have been found to be negatively phototactic despite their blindness and the complete absence of light in their environments (Tierney et al. 2015; Langille et al. 2018). These findings suggest that the ability to detect light may persist in some taxa, perhaps as an advantage to early cave colonizers or to serve for a yet unknown purpose once the organism is fully darkness-adapted (Langille et al. 2018). Selection tests on a LWS opsin in one of these subterranean beetles suggest the latter, as they did not result in any significant differentiation between habitats, and in fact strongly suggested that purifying selection was acting on the gene instead (Tierney et al. 2015). Similar observations have been made in the cave model species *A. mexicanus*, where transcription of opsins are evident during development but not necessarily in adult individuals (Langecker et al. 1993). These findings could very well be indicative of pleiotropic roles for LWS opsins in cave organism that deserve further attention. The possibility for the latter occurrence is also reflected in our results for the

amphipod *N. hrabei*. Both surface and cave populations of this species express a portion of the phototransduction pathway (Table 3), including identical opsin genes being transcribed in both cave and surface individuals. Due to an obvious lack of eyes, it is hypothesized that the presence of opsin proteins in this species is maintained for extraocular photoreception through alternate pathways (see Kelley and Davies [2016] for a review on non-visual light detection). In fact, behavioral studies have found that at least nine subterranean (and eyeless) species of *Niphargus* from Croatia, Italy, and Slovenia display negative phototactic behaviors (Borowsky 2011; Fišer et al. 2016). Previous studies investigating amphipod (*Gammarus minus*) cave and surface populations in the eastern USA also found that functional visual opsins were still present in aphotic cave populations (Carlini et al. 2013). However, the aforementioned study solely investigated opsins and did not verify whether the remainder of the phototransduction pathway had been transcribed. Although several studies have attributed the expression of functional visual opsins to pleiotropy, the possible roles that these opsins might be playing have not yet been subject of attention (Tierney et al. 2015). A recent study by Zanini et al. (2018) found two visual opsins being expressed in *Drosophila* proprioreceptors and presented evidence suggesting their involvement in cilium organization and mechanotransduction. It is therefore plausible that a similar locomotory mechanism is at play in cave arthropods, which would also explain the conservation of putative functional visual opsins in an environment completely devoid of light. Future prospects for the elucidation of these possibly pleiotropic roles are promising, as sequencing technologies continue to improve and become more affordable for non-model cave organisms.

Conclusions

The present study provides first insights into the evolution of vision loss in subterranean crustaceans from Molnár János Cave. We hypothesize that if vision loss has indeed occurred in recent cave colonizer *A. aquaticus*, it is likely due to down-regulation of visual opsins leading to reduced activation of the pathway that is still present in the transcriptome. Vision loss in recent surface colonizer *N. hrabei* was likely due to repressed expression or complete loss of eye development genes, rather than by the down-regulation of the phototransduction cascade's activator as in the hypothesized case of *A. aquaticus*. Although functional visual opsins were found being expressed in *N. hrabei*, determining whether these form part of an alternate photoreceptive pathway or play a role in a yet unknown function remains subject of ongoing and future studies. The transcriptomic data suggest that Molnár János Cave *A. aquaticus* might be capable of light-detection and perhaps have not fully lost their ability to see, in contrast to their blind Slovenian and Romanian cave conspecifics (Sket 1994). Differential gene expression/methylation analyses and whole genome sequencing projects currently being undertaken will undoubtedly provide additional insights into the mechanisms involved in vision loss within this system and other extreme aphotic environments.

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Supplementary data

Supplementary data available at *ICB* online.

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