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RESEARCH ARTICLE

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Standing genetic variation as a potential mechanism of novel cave phenotype evolution in the freshwater isopod, *Asellus aquaticus*

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Abstract

Novel phenotypes can come about through a variety of mechanisms including standing genetic variation from a founding population. Cave animals are an excellent system in which to study the evolution of novel phenotypes such as loss of pigmentation and eyes. *Asellus aquaticus* is a freshwater isopod crustacean found in Europe and has both a surface and a cave ecomorph which vary in multiple phenotypic traits. An orange eye phenotype was previously revealed by F_2 crosses and backcrosses to the cave parent within two examined Slovenian cave populations. Complete loss of pigmentation, both in eye and body, is epistatic to the orange eye phenotype and therefore the orange eye phenotype is hidden within the cave populations. Our goal was to investigate the origin of the orange eye alleles within the Slovenian cave populations by examining *A. aquaticus* individuals from Slovenian and Romanian surface populations and *Asellus aquaticus infernus* individuals from a Romanian cave population. We found orange eye individuals present in lab raised surface populations of *A. aquaticus* from both Slovenia and Romania. Using a mapping approach with crosses between individuals of two surface populations, we found that the region known to be responsible for the orange eye phenotype within the two previously examined Slovenian cave populations was also responsible within both the Slovenian and the Romanian surface populations. Complementation crosses between orange eye Slovenian and orange eye Romanian surface individuals suggest that the same gene is responsible for the orange eye phenotype in both surface populations. Additionally, we observed a low frequency phenotype of eye loss in crosses generated between the two surface populations and also in the Romanian surface population. Finally, in a cave population from Romania, *A. aquaticus infernus*, we found that the same region is also responsible for the orange eye phenotype as the Slovenian cave populations and the Slovenian and Romanian surface populations. Therefore, we present evidence that variation

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present in the cave populations could originate from standing variation present in the surface populations and/or transgressive hybridization of different surface phylogenetic lineages rather than de novo mutations.

KEYWORDS
cave animals, lineage hybridization, novel phenotype evolution, pigment, standing genetic variation, transgressive phenotype

1 | INTRODUCTION

Striking novel phenotypes, such as turtle shells, bat wings, and beetle horns, can be seen in a wide array of organisms. How do these novel phenotypes come about? Where does the phenotypic variation come from: is it environmental, genetic, or a combination of both? How much time does it take to evolve a novel phenotype, and if the same novel phenotype evolves multiple times, are the same or different mechanisms utilized?

There are several proposed mechanisms of how novel phenotypes evolve. First, new mutations could generate new phenotypes. Conversely, existing or standing genetic variation in a founding population might be advantageous in a new environment and then increase in frequency in the new environment. This has been documented in many model systems, including the three-spine stickleback (reviewed in Aguirre et al., 2022). Another potential mechanism of a how a novel phenotype evolves is that the normal function of heat shock protein (Hsp, a family of molecular chaperones that are produced by cells in response to stressful conditions) shields the uncovering of variation. Then when the heat shock protein function is impaired, novel phenotypes can result (reviewed in Zabinsky et al., 2019). Additionally, environmental variation could elicit the uncovering of different phenotypes through phenotypic plasticity (reviewed in Fox et al., 2019). In transgressive segregation, different species or populations interbreed and their hybrids might have extreme or novel phenotypes. This has been documented extensively in plants (reviewed in Mackay et al., 2021). Some of these mechanisms can be difficult to investigate or corroborate as they might require information about populations that are not extant, the frequency of alleles of interest might be very low in the population, or specific crosses might need to be set up which might be difficult or impossible in the laboratory environment.

An excellent set of species in which one can study the evolution of novel phenotypes is cave animals. Many novel phenotypes can be present including eye loss, pigmentation loss, longer appendages, and enhanced sensory systems. Though cave animals hold the potential to study the evolutionary mechanism of novel phenotypes, most cave animals do not have the tools or features necessary to investigate this question in a detailed manner with the exception being the cavefish, *Astyanax mexicanus*. For example, phenotypic plasticity has been examined in *A. mexicanus* where surface fish raised for 2 years in the dark conditions exhibited multiple cave-like phenotypes including increased fat accumulation and resistance to starvation (Bilandžija et al., 2020). Also, standing genetic variation has been implicated in feeding behaviors in *A. mexicanus* through the presence of the same *mc4r* allele in multiple cave populations (Aspiras et al., 2015). On the other hand, different mutations in the gene, *oca2*, appear to be responsible for albinism in different cave populations (Protas et al., 2006). Recently, hybridization between cave and surface populations has been demonstrated as an agent of evolution of cave specific phenotypes (Moran et al., 2022). And finally, Hsp90 as a capacitor of evolution in cavefish has also been demonstrated through the uncovering of eye size variation upon inhibition of Hsp90 (Rohner et al., 2013). Therefore, multiple evolutionary mechanisms leading to novel phenotypes have been observed within *A. mexicanus* and other cave animals hold the potential to explore these further.

One other cave animal that has more recently emerged as an eco–evo and evo–devo model is the crustacean, *Asellus aquaticus* (Lafuente et al., 2021; Protas & Jeffery, 2012). This freshwater isopod has multiple cave and surface populations which are mutually different in multiple phenotypic traits including eye and pigment loss. The genes responsible for eye and pigment loss are unknown but there are mapped regions responsible for several pigment and eye phenotypes (Bakovic et al., 2021; Protas et al., 2011) and a draft genome has recently been published (Bakovic et al., 2021). Regarding eye pigmentation in cave populations of *A. aquaticus*, for the population from Pivka Channel of Planina Cave in Slovenia, there are three mapped regions. One region is responsible for absence of all pigmentation, one is responsible for orange eye versus brown eye pigmentation, and one is responsible for red eye pigmentation (Protas et al., 2011). At this point, none of the genes responsible for these phenotypes have been identified. Interestingly, there appears to be two ways

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to achieve loss of eye pigment in this cave population. One way is via a single gene responsible for absence of pigmentation and the other is mutations at two different genes, one responsible for an orange eye phenotype and another for a red eye phenotype. In addition, the unpigmented phenotype appears to be epistatic to both orange eye and red eye phenotypes (Protas et al., 2011). Therefore, though the cave population appears to have mutations causing no pigment, orange eyes, and red eyes, the phenotype of the cave individuals is unpigmented (not orange eyes or red eyes) because orange eyes and red eyes only shows in the absence of the unpigmented genotype. It is unclear why the cave population contains multiple regions/genes responsible for pigmentation differences. Surprisingly, a population from a different part of the same cave, Rak Channel of Planina Cave, was found to share all three regions responsible for similar phenotypes (Re et al., 2018). One interpretation of the shared regions in the two cave populations, Pivka Channel and Rak Channel of Planina Cave, is that the variation could come from standing genetic variation from the founding surface population. However, information that could support this hypothesis such as presence of similar phenotypes or the same causative alleles in the surface population or additional, geographically distant, cave populations with similar regions responsible for similar phenotypes has been lacking. We set out to address this question of whether standing genetic variation could be a mechanism of evolution of cave morphological traits in *A. aquaticus*. We hypothesized that standing genetic variation was a mechanism of evolution of cave morphological traits and that the same morphological variation present within the previously examined cave populations could be found both within surface populations and within additional cave populations.

To do this, we decided to bring a new population of the cave ecomorph to the lab, *A. aquaticus infernus* from Romania. This population is geographically distant, almost 2000 km from the Planina Cave populations that have been previously examined, and is also ecologically very different. While *A. aquaticus* live in nonsulfidic waters in the Planina Cave, *A. a. infernus* inhabits the sulfidic groundwater aquifer in the Mangalia region by the shore of the Black Sea, including Movile Cave as well as old drinking wells and sulfidic springs in vicinity. Microbial biofilms consisting of chemoautotrophic sulfur oxidizing microorganisms produce food in situ, underground, independently of the surface photosynthetic food production. The presence of copious amounts of food in this subterranean ecosystem allows to the presence of an abundant and diverse aquatic and terrestrial invertebrate communities compared to other cave ecosystems (Brad et al., 2021). Previous work on this population has focused on adult morphology and phylogeography (Konec et al., 2015; Turk-Prevorčnik & Blejec, 1998).

To investigate the idea whether standing genetic variation is a possible mechanism of evolution of the cave phenotype of *A. aquaticus*, we focused on a particular trait, that is, orange eyes, uncovered within crosses of individuals from Pivka Channel of Planina Cave population with individuals from Slovenian surface populations. This orange eye phenotype is hidden within the two Planina Cave populations, masked by the phenotype of no pigment (Protas et al., 2011). We discovered that two different surface populations, one from Romania and one from Slovenia, after being kept and bred in the lab for several generations, yielded orange eye individuals. We investigated whether the genetic region responsible for the orange eye phenotype, masked by the unpigmented phenotype, in the Slovenian caves was also responsible for the orange eye phenotype in the two surface populations. In addition, a low-frequency eyeless phenotype was found in offspring between the Romanian and Slovenian surface populations. Finally, we examined whether the orange eye phenotype was present, but hidden, within the Romanian cave population, *A. a. infernus*, and then investigated whether the same region was also responsible in this population.

2 | MATERIALS AND METHODS

2.1 | Animals

A surface population from Slovenia, Rakov Škocjan (N45.794144°, E14.293132°), was collected in August of 2016 and raised in the lab over several generations (named here as Slovenian surface population). Also, a surface population from Romania, Turkish Bath (N43.820139°, E28.491083°), was collected in July 2018 and raised in the lab for several generations (named here as Romanian surface population). Individuals from another surface location in Romania, Limanu Bridge Stream (N43.48284°, E28.31515°), were collected in May 2021. Additionally, the cave ecomorph of *A. aquaticus infernus*, referred to here as a cave population, was collected from wells (N43.823219°, E28.567069° and N43.820767°, E28.572825°) in the Mangalia region in Romania in May 2021. Animals were raised as previously described by Protas et al. (2011), except algae pellets were used as a food source.

2.2 | Crosses

Several types of crosses were made. Cross Type 1 were Slovenian surface to Romanian surface crosses where one parent had orange eyes and the other brown eyes (Figure 2A). Cross Type 2 were complementation crosses of orange eye individuals derived from the Slovenian

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surface population and orange eye Romanian surface individuals (Figure 3A). Cross Type 3 was a cross between a product of one of the crosses from Cross Type 2 with an *A. a. infernus* male (Figure 4A). Cross Type 4 were crosses of surface Romanian individuals with *A. a. infernus* males (Figure 5A,B). Cross Type 5 was a cross of an F_4 from the Rak Channel of Planina Cave to a surface individual with an *A. a. infernus* male (Figure 6A). All described crosses were raised at 12°C except some of the Type 5 crosses were raised at room temperature.

For Cross Type 1, Slovenian surface to Romanian surface crosses were generated. The goal of these crosses was to see if the region known to be responsible for orange eyes in the Planina Cave was also responsible for orange eyes in both surface populations. The reason for mapping the phenotype of orange eye in crosses between surface populations, rather than within a surface population, is that there is more genetic variation to be used as genetic markers between surface populations than within a single surface population. First, two orange eye males from the Slovenian surface population were crossed to two brown eye females from the Romanian surface population (Figure 2A). Then, sibling F_1 individuals were intercrossed generating F_2 individuals. A total of 48 individuals were generated for phenotyping and genotyping. Similarly, an orange eye female from the Romanian surface population was crossed to a brown eye male from the Slovenian surface population. In another cross, an orange eye Romanian male was crossed to a brown eyed female from the Slovenian surface population. For each cross, sibling F_1 individuals were intercrossed, generating F_2 individuals. A total of 36 individuals were generated for phenotyping and genotyping.

Cross Type 2 were complementation crosses (Figure 3A) which can yield information about whether the same gene or different genes are responsible for similar phenotypes. The most straightforward complementation cross would have been an orange eye individual from the Romanian surface population crossed to an orange eye individual from the Slovenian surface population. However, the single tank with orange eye Slovenian individuals had perished. So, instead, we used an orange eye F_2 female or an orange eye F_4 male, obtained by crossing an orange eye Slovenian surface individual to a brown eye Romanian surface individual, and crossed them to an orange eye Romanian surface male or to two orange eye Romanian surface females, respectively. A total of three complementation crosses were generated (the latter two using the same male) with a total of 37 offspring.

Cross Type 3 describes an eyeless F_3 female, from one of the above-described Slovenian surface to Romanian surface crosses, crossed to an *A. a. infernus* male generating 30 offspring (Figure 4A). The goal of this cross was a modified complementation cross to examine whether the eyeless phenotype from the surface crosses would complement with the eyeless phenotype in the *A. a. infernus* individuals. Note: to be considered a true complementation cross, the eyeless phenotype of both would have to be encoded by a single gene which cannot be confirmed at this time.

Cross Type 4 were crosses between surface Romanian individuals and *A. a. infernus* cave individuals. The goal of these crosses was to see if the same region and same gene responsible for the orange eye phenotype in the Romanian surface population were also responsible in the *A. a. infernus* population. Crosses were generated using Romanian surface females that had orange eyes or surface females that had brown eyes but were in a tank that contained orange eye individuals, and although brown were likely heterozygous for the orange eye allele. These surface females were crossed to *A. a. infernus* males generating F_1 individuals (Figure 5A,B). Four crosses using orange eye females generated a total of 59 offspring. Four crosses were generated with brown eye females generating a total of 140 offspring.

Cross Type 5 utilized a brown eye F_4 female, from intercrossed individuals made by crossing a Rak Channel of Planina Cave individual to a Slovenian surface individual, crossed to an *A. a. infernus* male, generating 11 offspring. The goal of this cross was to set up a modified complementation cross between the Rak Channel of Planina Cave with *A. a. infernus* to investigate if the same gene was responsible for the orange eye phenotype in both populations.

2.3 | Phenotyping and imaging

All individuals were phenotyped, that is, evaluated for the presence of orange or brown eye pigment, either achieving adult size of 5 mm or at death/dying if they died or were looking sickly before reaching adulthood. The orange eye phenotype is detectable even before hatching and therefore animals at all ages can be phenotyped for orange versus brown eyes. The eye was used to phenotype as head/body pigment of orange eye individuals could be variable ranging from orange to orangish brown. Phenotyping was performed using a Leica S8 Apo microscope with LAS (Leica) Core Software.

2.4 | Genotyping

DNA was extracted from ethanol-preserved whole bodies of animals or fresh pereopod tissue from F_2 individuals or parental individuals (Romanian or Slovenian surface populations) using QIAamp DNA Micro Kit or QIAamp DNA

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TABLE 1 Genetic markers used in the genes *nckx30* and *disconnected (disco)*

Genetic marker	Genetic marker sequence	Forward (*F*) and reverse (*R*) primer sequence
nckx30 part 1	F_2 of Slovenian surface X Romanian surface:	*F*: 5′-TCCTCCGAGATCTGCAACTTCTCTA-3′
	5′-GCCTTGGGCG[C/T]CGTCGCGTTCA-3′	*R*: 5′-GTCTTCGCTTGTCCAAATGACGATA-3′
	F_4/*A. a. infernus* complementation cross:	same as above
	5′-TCCGTGAA[A/T]ATGGAGGG-3′	
nckx30 part 2	F_1 of Romanian surface X *A. a. infernus*:	*F*: 5′-ATCGTCGCGTTGATATCGTTTTA-3′
	5′-TACAATTACGAGGATTTTT[T/-] GCTGTGTAACCTAATT-3′	*R*: 5′-TACAGGAAGTGCACAATTGATCC-3′
disco	F_4/*A. a. infernus* complementation cross:	*F*: 5′-AACCGCCATTCTGCTAATCC-3′
	5′-GGCAACCC[T/A]GTTAGAGG-3′	*R*: 5′-CGCTATTCATGCTGTCTTCCA-3′

Note: The fragment *nckx30 part 1* was used to genotype the F_2 crosses generated between Slovenian and Romanian surface populations. In the parenthesis within the fragment sequence, C marks the Slovenian surface allele while T marks the Romanian surface allele. The fragment *nckx30 part 1* was also used to genotype the offspring of the F_4 female (Rak Channel of Planina Cave × Rakov Škocjan) to *A. a. infernus* male. In the parenthesis within the fragment sequence, A marks both the Slovenian cave allele and the *A. a. infernus* allele, while T marks the Slovenian surface allele. The fragment *nckx30 part 2* was used to genotype the F_1 crosses generated between Romanian surface individuals and *A. a. infernus* cave individuals. In the parenthesis within the fragment sequence, "–" indicates a missing nucleotide. Therefore, one allele is one nucleotide longer than the other allele. Two possible genotypes were present in the F_1 individuals genotyped, either homozygous for the longer allele or heterozygous for the short and long allele which resulted in mixed sequence. Disconnected (*disco*) was also used to genotype the F_4 (Rak Channel of Planina Cave × Rakov Škocjan) to *A. a. infernus* cross. In the parenthesis within the fragment sequence, T marks the Slovenian cave allele, while A marks both the *A. a. infernus* cave allele and the Slovenian surface allele.

Mini Kit (Qiagen). Polymerase chain reaction (PCR) was performed using GoTaq Green Master Mix (Promega) with pairs of primers for *nckx30* and *disconnected (disco)* (Table 1). The PCR protocol that was used was 95°C for 5 min, then, 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and finally 72°C for 10 min. A 1.5% agarose gel with Sybr Safe solution (Invitrogen) was used to visualize PCR products. The PCR products were purified with ExoSAP-IT (Affymetrix). For all markers, only the forward primers were used for Sanger sequencing at MCLab. Sequences were visualized and edited using Geospiza FinchTV software (www.geospiza.com/finchtv).

2.5 | Identification of genetic markers

To obtain a genetic marker that could be used to genotype the F_2 individuals from the Slovenian X Romanian surface crosses four Slovenian surface and four Romanian surface individuals were sequenced for a piece of *nckx30* which marks the region responsible for orange eye in the Slovenian cave populations (Protas et al., 2011; Re et al., 2018). SNPs were found that were fixed within the individuals examined in each population and one was identified to use to genotype the F_2 offspring (Table 1).

To genotype the F_1 crosses resulting from Romanian surface individuals and *A. a. infernus* individuals, the surface females needed to be heterozygous for a difference in *nckx30*, the gene to be genotyped. If heterozygous, that marker could be tracked within their F_1 offspring and could be examined to see if there were an association between the orange eye phenotype and genotype of *nckx30* within the F_1 individuals. The original part of *nckx30* used as a marker for the intercrosses between the two surface populations was not heterozygous in the surface females so could not be used. Therefore, other parts of the same gene were investigated, and one part was heterozygous in the mothers of both F_1 crosses (Table 1). This *nckx30* "part 2" should mark the same region of the genome as *nckx30* "part 1" and therefore should also be able to test for linkage of the region responsible for orange eye in the Slovenian caves.

To genotype the offspring of the F_4 individual from Rak Channel of Planina Cave and Rakov Škocjan with the *A. a. infernus* male, previously described markers in *nckx30* and *disconnected* were used (Table 1; Re et al., 2018).

2.6 | Statistical tests

Fisher's exact tests were performed for each genetic marker and its associated trait to explore whether a significant association between genotype and phenotype exists. The strength of association was assessed using Cramér's V, which varies from 0 (no association) to 1 (complete association). All calculations were performed in R 4.0.2. (R Development Core Team 2022).

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3 | RESULTS

3.1 | Orange eye alleles are present within Slovenian and Romanian surface populations and map to the same region as the orange eye alleles from the Slovenian cave populations

After being raised in the lab for multiple generations, surface individuals from both populations in Romania and Slovenia have shown individuals that have an orange eye phenotype, similar to that found in backcross and F_2 individuals from the Slovenian cave populations crossed to their nearest surface populations (Figure 1). For Cross Type 1, F_2 crosses generated either from orange eye Romanian surface individuals to brown Slovenian surface individuals or orange eye Slovenian surface individuals to brown Romanian surface individuals resulted in F_2 individuals that had either orange eyes or brown eyes (Figure 2b–e). F_2 individuals were genotyped for *nckx30*, a gene that was previously shown to mark the region responsible for orange eye pigment in the Planina Cave populations but is not the actual gene responsible for the orange eye phenotype (Protas et al., 2011). The genotype of *nckx30* and the phenotype of orange eyes or brown eyes and genotype of *nckx30* were significantly associated for both reciprocal crosses (Table 2) indicating

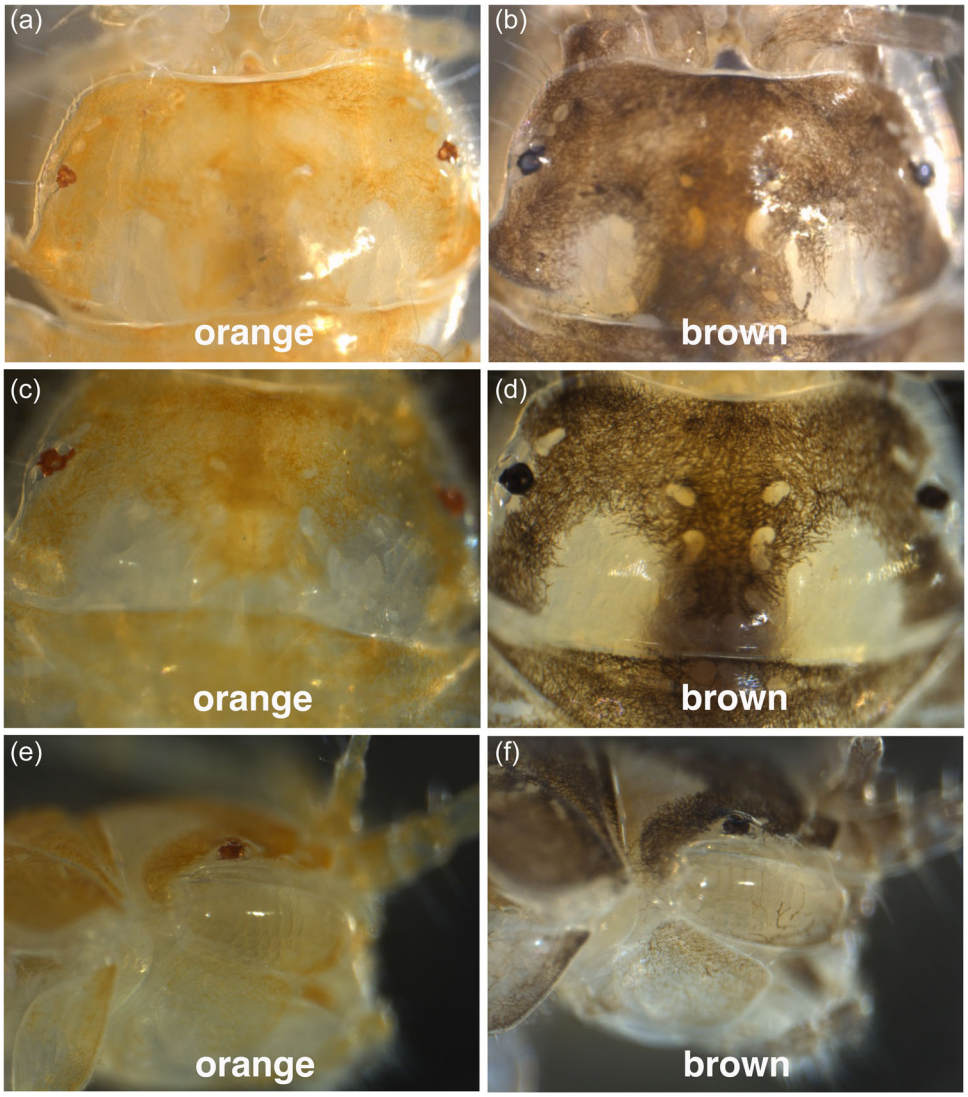

FIGURE 1 Orange eye phenotype in the surface populations is similar to the orange eye phenotype revealed within crosses with the Slovenian cave populations and Slovenian surface population. (a) An orange eye F_2 individual resulting from Rak Channel of Planina Cave and Slovenian surface (Rakov Škocjan) cross (Re et al., 2018). (b) A brown eye F_2 individual resulting from Rak Channel of Planina Cave crossed to Slovenian surface (Re et al., 2018). (c) An orange eye Romanian surface individual. (d) A brown eye Romanian surface individual. (e) Profile of an orange eye Romanian surface individual. (f) Profile of a brown eye Romanian surface individual. [Color figure can be viewed at wileyonlinelibrary.com]

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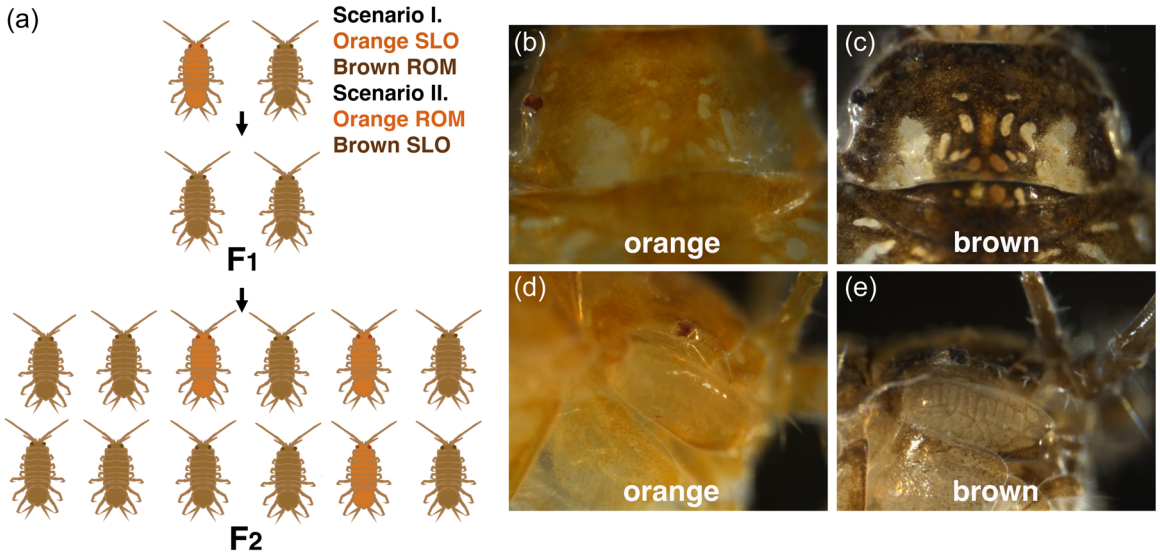

FIGURE 2 Romanian to Slovenian surface population crosses used to map the region responsible for orange eye in both surface populations. (a) Scenario I: Orange eye Slovenian (SLO) surface individual was crossed to a brown eye Romanian (ROM) surface individual generating brown eye F_1 individuals. These were then crossed to each other generating F_2 individuals that were either brown eye or orange eye. Scenario II: The reciprocal cross was also performed; an orange eye Romanian (ROM) surface individual was crossed to a brown eye SLO individual. These brown eye F_1 individuals were also crossed together to generate F_2 individuals that were both brown eye and orange eye. (b) and (d) An example of an orange eye F_2 from the cross where the orange eye individual was from Romania. (c) and (e). An example of a brown eye F_2 from the cross where the orange eye individual was from Romania. Brown eye and orange eye F_2 individuals from the reciprocal cross are not pictured here but were similar in phenotype to that shown in (b)–(e). Schematic courtesy of Dennis Sun. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Genotype of *nckx30* is associated with orange eye color in both Slovenian and Romanian surface populations

F_2 individuals from orange Slovenian × brown Romanian				**Fisher's exact test**	**Cramér's V**	**F_2 individuals from orange Romanian × brown Slovenian**				**Fisher's exact test**	**Cramér's V**
nckx30	SS	RS	RR			*nckx30*	SS	RS	RR		
Orange	14	1	0	$p < 0.001$	0.953	Orange	0	1	14	$p < 0.001$	0.945
Brown	0	16	17			Brown	6	15	0		

Note: F_2 individuals from the orange eye Slovenian and brown eye Romanian cross and vice versa were genotyped. Three genotypes were possible: homozygous for the Slovenian allele (SS), homozygous for the Romanian allele (RR) or heterozygous with one copy of the Romanian allele and one copy of the Slovenian allele (RS).

that the same region responsible for the orange eye phenotype in the Slovenian cave populations is likely responsible for the orange eye phenotype in the Slovenian surface and Romanian surface populations.

Next, to investigate if the same gene was responsible for the orange eye phenotype in the Slovenian surface and Romanian surface populations as in the Slovenian cave populations, complementation tests were performed between orange eye F_2 or F_4 individuals (from the cross of Slovenian surface orange eye X Romanian surface brown eye) crossed to Romanian orange eye individuals (Cross Type 2). As expected, the F_2 and F_4 orange eye individuals were homozygous for the Slovenian allele at *nckx30*, the Romanian orange eye individuals were homozygous for the Romanian allele, and the 11 offspring genotyped (a subset of the offspring from the three crosses) were all heterozygous for the Romanian and Slovenian alleles (Figure 3 and Table 3). Summing the three complementation crosses, 37 offspring were produced, and all had orange eyes. Therefore, it is likely that the same gene is responsible for the orange eye phenotype in the Romanian and Slovenian surface populations.

3.2 | Eyeless phenotype generated in crosses between the Slovenian and Romanian surface populations

An eyeless phenotype, no ommatidia and no eye pigment on at least one side of the head, showed up

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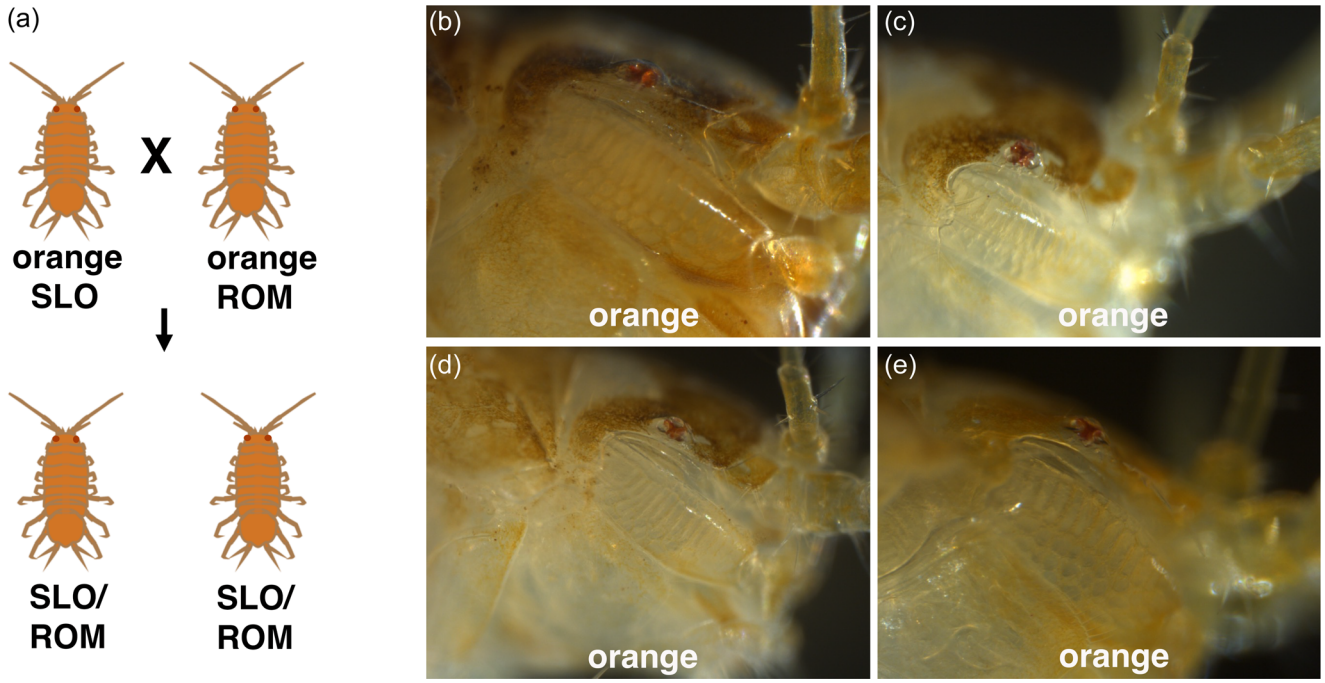

FIGURE 3 Phenotypes of complementation cross between orange eye individuals from Slovenian and Romanian surface populations. (a) Orange eye F_2 female (orange SLO) (from orange eye surface Slovenian X brown surface Romanian cross) crossed to orange eye surface Romanian individual (orange ROM) generating hybrids with one Slovenian allele for orange eye and one Romanian allele for orange eye. (b) Orange eye F_2 female (orange SLO, mother of d and e). (c) Orange eye Romanian male (orange ROM, father of d and e). (d) and (e) Offspring of a cross between (b) and (c), both showing an orange eye phenotype. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Complementation crosses between orange eye individuals from Slovenian and Romanian surface populations

Female parent	Genotype of female parent	Male parent	Genotype of male parent	Offspring phenotype	Offspring *nckx30* genotype
Orange SLO	SS	Orange ROM	RR	2 orange	2 RS
Orange ROM	RR	Orange SLO[a]	SS	16 orange	5 RS
Orange ROM	RR	Orange SLO[a]	SS	19 orange	4 RS

Note: Orange SLO (F_2 or F_4 individuals from orange eye surface Slovenian X brown eye surface Romanian cross) crossed to Orange ROM (orange eye individuals from Romanian surface population). The first cross in the table is the one shown in Figure 3. Only a subset of the offspring of the complementation crosses were genotyped (a total of 11 individuals) but all showed the expected genotype of one Romanian surface allele and one Slovenian surface allele (RS).

Abbreviations: R, Romanian surface allele, S, Slovenian surface allele.

[a]The same male parent was used in both crosses.

infrequently within the intercrossed individuals from Slovenian and Romanian surface populations, first observed in the F_3 generation, but not observed within either surface population used for this cross. A total of seven individuals, derived from two separate orange eyed Slovenian individuals crossed to two different brown Romanian individuals showed this eyeless phenotype so far. To get an estimate of the frequency of this phenotype, we measured all animals derived from these crosses present at the F_3, F_4, and F_5 generations at a given time and there were 2 eyeless individuals and 110 eyed individuals. Individuals showed an eyeless phenotype, no eye pigment and no ommatidia, on at least one side of the face (Figure 4). Some individuals showed variation in their two eyes, such as one eye had no eye pigment and no ommatidia, but the other eye had ommatidia but no eye pigment (Figure 4d). To compare the genetic basis of this possibly transgressive phenotype to that of the eyeless phenotype in the *A. a. infernus* cave population, Cross Type 3 was performed, and we crossed an individual with no ommatidia and no eye pigment on one side of the face and ommatidia with no eye pigment on the other side of the face with an *A. a. infernus* individual. Offspring all had ommatidia (Figure 4). This does not mean that complementation occurred as we do not

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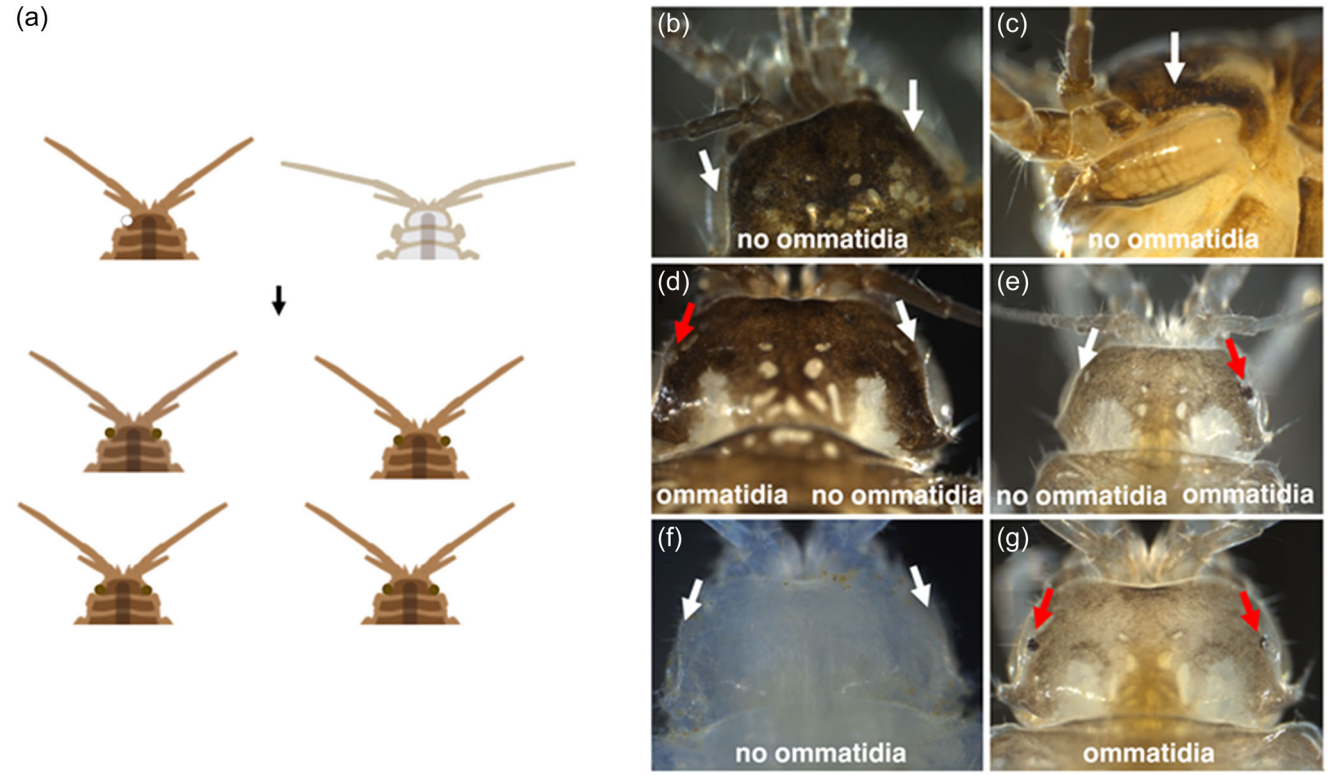

FIGURE 4 Eyeless individuals were present at a low frequency in the crosses of orange eye Slovenian surface individuals to brown eye Romanian surface individuals as well as one individual from another Romanian surface location. (a) Schematic of a cross between a surface intercross individual with no pigmented eye spots and ommatidia on only one side of the head crossed to an *A. a. infernus* cave individual. (b) A head of an eyeless individual from a surface intercross with no ommatidia on both sides of its head (white arrows). (c) A profile of an eyeless individual with no pigmented eye spot and no ommatidia on the left-hand side (white arrow) and a slightly pigmented eye spot and ommatidia on the right-hand side of its head (the latter not shown). (d) A head of another eyeless individual with ommatidia but no pigmented eye spot on the left side (red arrow) and with no pigmented eye spot and no ommatidia on the right side of its head (white arrow). (e) An eyeless individual, no ommatidia on the left side (white arrow) but ommatidia and pigmented eye spot on the right side (red arrow), from a lab-bred tank of the Limanu Bridge Stream Romanian surface location. (f) An *A. a. infernus* cave individual. (g) Offspring of the individual shown in D and an *A. a. infernus* cave male (not shown) with eye pigment and ommatidia in both eyes (red arrows).

know whether either eyeless phenotype is encoded by a single gene.

In addition to the seven eyeless individuals observed in the surface intercrossed individuals, we found a single eyeless animal within a lab-bred tank of Limanu Bridge Stream surface individuals which is located near the Romanian surface population used for the surface intercross. In this eyeless individual, one side of the head had no ommatidia and no eye pigment and the other side of the head had ommatidia and eye pigment (Figure 4e).

3.3 | Orange eye alleles are present within the *A. a. infernus* cave population

Because the orange eye phenotype was present within the Romanian surface population, we wondered if the *A. a. infernus* cave population was similar to the Slovenian cave population and also had the orange eye alleles present but the orange eye phenotype was masked by the unpigmented phenotype. To address this, we set up Cross Type 4. First, we crossed orange eye Romanian surface individuals to *A. a. infernus* cave individuals (Table 4 and Figure 5). Of the four crosses that successfully produced offspring, all offspring had orange eyes (Table 4). Other crosses were set up with brown eye individuals, likely heterozygotes for the orange eye allele (Table 4 and Figure 5). Of four crosses that successfully produced offspring, there were both brown eye and orange eye offspring. One possible interpretation of the phenotypes resulting from these crosses is that the *A. a. infernus* population does have the orange allele, but the phenotype is masked by the unpigmented phenotype (recessive epistasis) and that what we are essentially seeing is noncomplementation through the presence of orange eye F_1

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individuals as all these individuals should be heterozygous for the no pigment allele (and therefore will be pigmented) and have the chance to show the orange eye phenotype. For example, gene A is responsible for the unpigmented phenotype and A_1A_1 encodes the unpigmented phenotype, and A_1A_2 or A_2A_2 encodes brown eyes. Gene B is responsible for the orange eye phenotype and B_1B_1 encodes orange eyes and B_1B_2 or B_2B_2 causes brown eyes. If the animal's genotype is A_1A_1, it does not matter what the genotype is at gene B because the individual will still be unpigmented. However, if at gene A there is either a A_1A_2 or A_2A_2 genotype, then the genotype at gene B does matter and B_1B_1 individuals will have orange eyes and B_1B_2 or B_2B_2 will have brown eyes. Orange eye surface females ($A_2A_2B_1B_1$) crossed to *A. a. infernus* males ($A_1A_1B_1B_1$) should all be $A_1A_2B_1B_1$ and have orange eyes. Heterozygous for orange eye surface females ($A_2A_2B_1B_2$) crossed to *A. a. infernus* males ($A_1A_1B_1B_1$) should be half brown eyes ($A_1A_2B_1B_2$) and half orange eyes ($A_1A_2B_1B_1$).

TABLE 4 Phenotypes of F_1 crosses between surface Romanian individuals and *A. a. infernus* cave individuals

Phenotype of cave male	Phenotype of surface female	No. of brown F_1 offspring	No. of orange F_1 offspring
Unpigmented	Orange	0	12
Unpigmented	Orange	0	5
Unpigmented	Orange	0	28
Unpigmented	Orange	0	14
Unpigmented	Brown*	19	22
Unpigmented	Brown*	12	10
Unpigmented	Brown*	12	4
Unpigmented	Brown*	39	22

Note: Surface Romanian individuals had either orange or brown eyes. Brown* = phenotypically brown eyes but genotypically likely has one brown eye allele and one orange eye allele. Orange = orange eyes. Note that there were crosses with brown eye surface females that only yielded brown eye offspring but those are not included in this table.

To test this idea, we decided to genotype the F_1 generation, as historically F_2 individuals had been difficult to generate for *A. a. infernus*. F_1 offspring from crosses containing both orange eye and brown eye individuals were genotyped and phenotyped and a significant association was found between the phenotype of orange eye and the genotype at *nckx30* (Table 5). In sum, it appears that the same region is responsible for orange eye in the Pivka Channel of Planina Cave population, Rak Channel of Planina Cave population, Slovenian surface population, Romanian surface population, and *A. a. infernus* cave population.

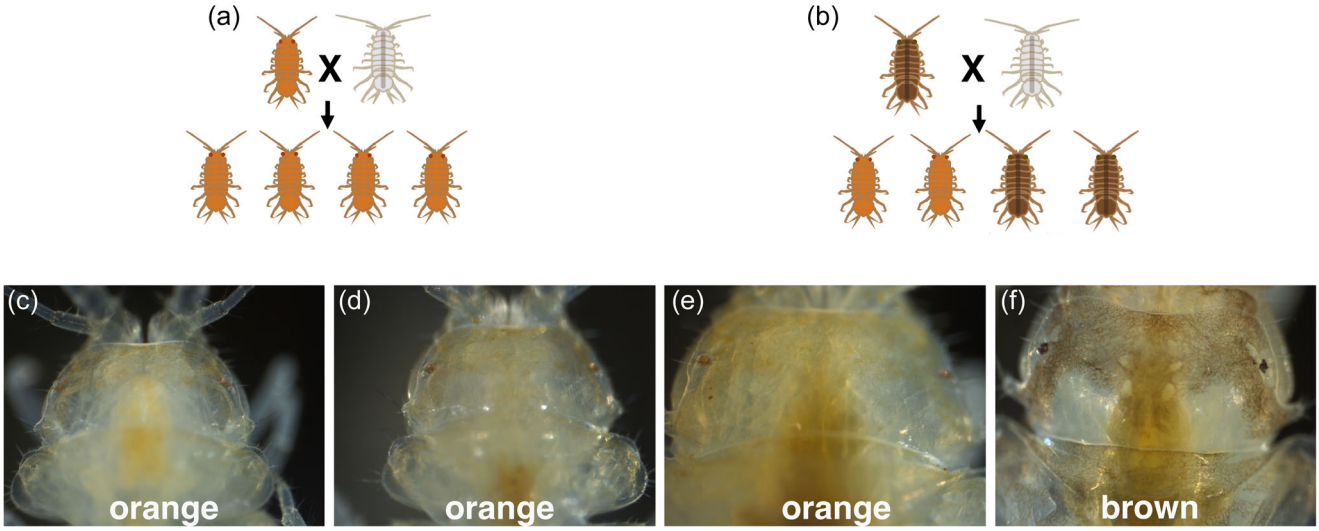

FIGURE 5 F_1 crosses of *A. a. infernus* cave individuals with brown eye and orange eye Romanian surface individuals resulted in orange eye offspring. For the following genotypes, the model from the Planina Cave is used where gene A is responsible for pigment or no pigment and gene B is responsible for orange eye or brown eye. Two copies of the cave no pigment allele (A_1A_1) result in no pigment (regardless of the genotype at gene B) and two copies of the cave allele at gene B result in orange eye (B_1B_1), only if there is at least one surface allele at gene A (Protas et al., 2011). (a) Schematic of a cross of an orange eye surface individual ($A_2A_2B_1B_1$) crossed to a *A. a. infernus* cave individual ($A_1A_1B_1B_1$). (b) Schematic of cross of a presumably heterozygous for orange eye but phenotypically brown eye individual ($A_2A_2B_1B_2$) crossed to a *A. a. infernus* cave individual ($A_1A_1B_1B_1$). (c, d) Orange eye offspring from a cross shown in A ($A_1A_2B_1B_1$). (e) Orange eye offspring from a cross shown in B ($A_1A_2B_1B_1$). (f) Brown eye offspring from a cross shown in B ($A_1A_2B_1B_2$). [Color figure can be viewed at wileyonlinelibrary.com]

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TABLE 5 The genotype of *nckx30* was associated with the phenotype of orange eye pigment in F_1 crosses between brown eye surface females heterozygous for orange eye crossed to *A. a. infernus*.

Eye color	Heterozygous	Homozygous	Fisher's exact test	Cramér's V
Orange	21	0	$p < 0.001$	0.907
Brown	2	18		

Note: A total of 13 and 28 individuals from two separate crosses were genotyped for *nckx30*. These two crosses originally gave more offspring but we excluded those that died very early and one individual without eye pigment.

TABLE 6 Genotypes of cross between F_4 (original parents Rak Channel of Planina Cave and Slovenian surface Rakov Škocjan) and *A. a. infernus* individual

Eye color	IS	IP	Fisher's exact test	Cramér's V
disconnected				
Pigment (either brown or orange)	6	0	$p = 0.002$	1
No pigment	0	5		
nckx30				
Brown	5	0	$p = 0.167$	1
Orange	0	1		
No pigment	2	3		

Note: Offspring were five brown eye, five no pigment, and one orange eye. "I" indicates the *A. a. infernus* allele, "S" is the Slovenian surface allele, and "P" is the Slovenian Rak Channel of Planina Cave allele. *Disconnected* shows a significant association between phenotype of no pigment/pigment (orange eye + brown eye) and genotype at *disconnected*. The Fisher's exact test for *nckx30* compared only those that were orange eye or brown eye and was nonsignificant. However, the one orange eye individual does have the genotype IP as expected and all brown eye individuals have the genotype IS, as expected. Thus, the nonsignificant result is most likely due to low sample size. The no pigment individuals are expected to have both possible genotypes, IS or IP, as half of the no pigment individuals are expected to have the genotype for orange eye though phenotypically they are unpigmented due to epistasis.

Ideally, we would have set up a complementation cross between orange eye F_2 individuals from the Planina Cave and orange eye F_2 individuals from *A. a. infernus* crosses to test if the same gene was responsible for the orange eye phenotype in the Planina Cave and *A. a. infernus* populations. Though we did not have the above animals, we did have a brown eye F_4 female from crosses with the Slovenian surface and Rak Channel of Planina Cave population. We crossed it to an *A. a. infernus* male setting up Cross Type 5. The offspring were five with brown eyes, five with no pigment, and one with orange eyes (Table 6 and Figure 6). We hypothesized that the brown F_4 mother was heterozygous for the orange eye allele and heterozygous for the no pigment allele and her offspring were showing noncomplementation as there were both unpigmented and orange eye phenotypes. To test this, we genotyped all 11 offspring for two genetic markers, one in *disconnected* which marks presence versus absence of pigment in the Planina Cave populations and again *nckx30* which marks orange eye pigment (Protas et al., 2011; Re et al., 2018). If the same genes were responsible for no pigment and orange eye pigment in the *A. a. infernus* population as compared to the Planina Cave population, and no pigment is epistatic to orange eye pigment in *A. a. infernus* as it appears to be in the Planina Cave, we expected that the orange eye individual would have one copy of the Planina Cave allele for *nckx30*, and one copy of the surface allele for *disconnected*. (For all of the offspring they should have one copy of the *A. a. infernus* allele for both *nckx30* and *disconnected* as one parent is an *A. a. infernus* individual). The genotype of the orange eye individual was as expected (Table 6 and Supporting Information: Table S1). In addition, we expected that the brown eye individuals would have one copy of the surface allele for *nckx30* and one copy of the surface allele for *disconnected;* this was the case for all five brown eye individuals. We expected that the unpigmented individuals would have one copy of the Planina Cave allele for *disconnected* and either a copy of the surface allele or a copy of the Planina Cave allele for *nckx30*; all five unpigmented individuals did have one copy of the Planina Cave allele for *disconnected* and three of them had the Planina Cave allele for *nckx30* and two of them had one copy of the Slovenian surface allele. Though there were only 11 individuals, the model of two genes,

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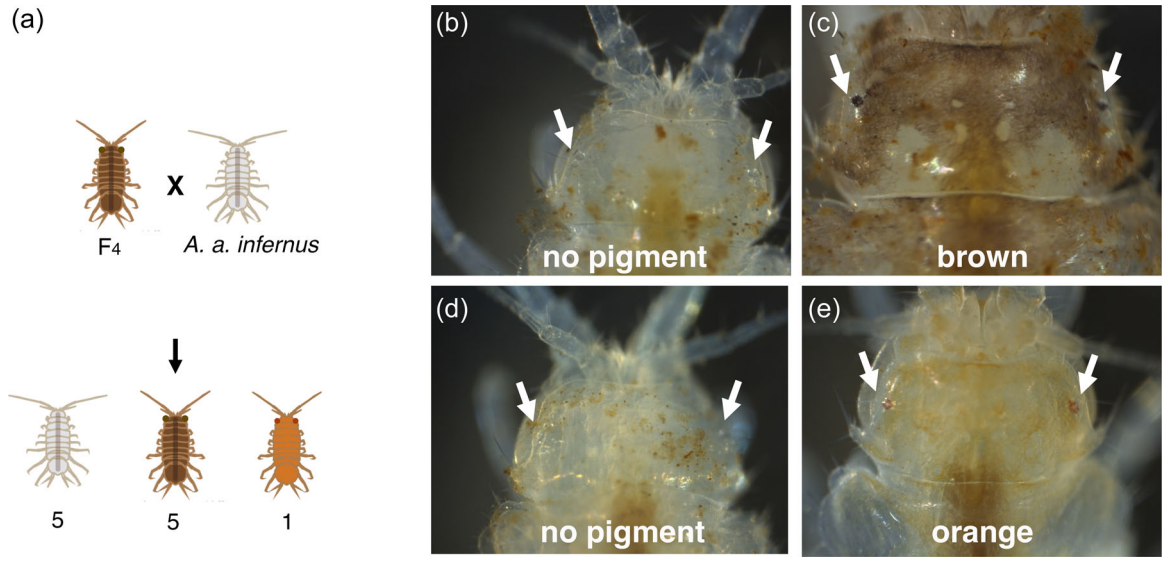

FIGURE 6 Cross between F_4 individual derived from the Rak Channel of Planina Cave to *A. a. infernus* male showed both orange and unpigmented individuals. (a) Schematic showing that a F_4 female ($A_1A_2B_1B_2$) from original parents of a Rak Channel of Planina Cave male to a Slovenian surface (Rakov Škocjan) female was crossed to an *A. a. infernus* male ($A_1A_1B_1B_1$). Arrows show pigmented or unpigmented eye spots. The cross gave 11 offspring: one orange eye ($A_1A_2B_1B_1$), five brown eyes ($A_1A_2B_1B_2$), and five unpigmented ($A_1A_1B_1B_2$ or $A_1A_1B_1B_1$). (b, d) No pigment individual. (c) Brown eye individual. (e) Orange eye individual. Note, all animals, even unpigmented ones, have organic debris on their exoskeleton which looks like brown spots and should not be confused with pigment. [Color figure can be viewed at wileyonlinelibrary.com]

one responsible for albino, one responsible for orange eyes, and albino epistatic to orange eyes fits the above genotype for all individuals (Table 6 and Supporting Information: Table S1). However, due to small sample size, the genotype at *nckx30* and the phenotype of orange eye and brown eye were not significantly correlated. The genotype at *disconnected* and the phenotype of no pigment versus pigment were significantly correlated.

4 | DISCUSSION

The same region known to be responsible for the orange eye phenotype in the Pivka and Rak Channels of Planina Cave populations was also responsible for the orange eye phenotype in both Slovenian and Romanian surface populations. This supports the idea that standing genetic variation in an ancestral surface population can be the medium of evolutionary change, which has been widely supported in studies of similar systems including sticklebacks and cichlids (Aguirre et al., 2022; Urban et al., 2021). Though we do not know that the orange eye allele was present in the founding surface population of the cave populations, its presence in the current surface populations indicates that it likely could have been present then as well. However, one possibility that we cannot exclude is that the orange eye allele originated in the cave populations and then was reintroduced into the surface populations by hybridization between the cave and surface ecomorphs. However, this possibility is less parsimonious and thus less likely as it would necessitate multiple de novo mutations of the orange eye allele.

It is unclear how or why this variation would be preserved in the surface population. Knowledge of the gene responsible for the orange eye phenotype will be necessary to elucidate possible reasons that it was not eliminated from the surface population. However, pigment variation has been documented within surface populations of *A. aquaticus* and has been associated with different environmental conditions in surface habitats (Bakovic et al., 2021; Hargeby et al., 2004, 2005; Lürig et al., 2019). Furthermore, though our focus has been on the genetic basis of pigmentation, pigmentation within *A. aquaticus* has also been demonstrated to be highly phenotypically plastic and can be affected by diet (Lürig & Matthews, 2021; Lürig et al., 2019). In our experiments, animals were raised in similar environmental conditions with the same food source with the goal of minimizing variation due to environmental variables. Also, we attempted to phenotype animals as adults though some we had to genotype some earlier due to poor survival. Fortunately, the orange eye phenotype can be differentiated from the brown eye phenotype already

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towards the end of embryogenesis. We phenotyped by eye color as the head and body color was more variable; orange eyed individuals can have orange head and body pigmentation or orangish/brown head and body pigment. Because our lab conditions are not identical to the natural environment, one unanswered question is whether animals that show an orange eye phenotype in the lab also would show an orange eye phenotype in their natural habitat. Interestingly, the orange eye phenotype came up in multiple tanks of the Romanian surface population but only in a single tank in the Slovenian population which might mean that it is a more common allele in certain surface populations. This could stem from the proximity of different habitats where lighter pigmentation might be advantageous such as the stonewort habitats in Sweden (Bakovic et al., 2021; Hargeby et al., 2004). One potential difference here is that we see a different color (a qualitative difference) rather than lighter brown pigmentation (a quantitative difference). It will be interesting to examine whether orange eyed individuals can be found in surface environments, or the variation found in surface environments is from amounts of brown pigment rather than different colors.

As mentioned above, the orange eye phenotype can be detected toward the end of embryogenesis. Normally, in *A. aquaticus*, the eye first shows pigmentation of a reddish/orangish color (Mojaddidi et al., 2018). Then, the pigment darkens over time such that the hatchling has brown pigmentation. Pigments present in the integument of *A. aquaticus* have been described as ommochromes (Needham & Brunet, 1957). Interestingly, another phenotype that can be found in backcross individuals between the Pivka Channel of Planina Cave and surface individuals is red eyes and has mapped to a different location than the region responsible for orange eyes (Protas et al., 2011). Backcross individuals from the Pivka Channel of the Planina Cave that have both the orange eye and red eye alleles were either unpigmented or had very faint red eyes. Therefore, it is possible that the red eye alleles are defective in generation of one type of pigment and the orange eye alleles are defective in another pigment and that these two pigments together generate a brown color. Conversely, the red eye allele and orange eye allele could perturb different steps within the same pigment synthesis pathway. Future work investigating the pigments in *A. aquaticus*, and their time course of deposition during animal's ontogeny, will be helpful in determining the developmental basis of the pigmentation differences generated through the orange eye and red eye alleles.

A byproduct of crossing together the two surface populations was the generation of a novel eyeless phenotype that has not been observed within either parental surface population although we found a similar phenotype in a single individual from a lab-bred tank of a nearby surface location. One possibility is that standing variation could be responsible for the eyeless phenotype which is supported by the existence of the single eyeless individual from the nearby surface location. The eyeless individuals within the surface intercross could also result from standing variation from within either of the parental surface populations used to generate the intercross. However, it is surprising that eyeless individuals have not been seen within either parental surface population even though they have been bred in the lab for four or more years. Such inbred lab populations would be expected to express rare recessive alleles. An alternative hypothesis to explain the eyeless phenotype found within the surface intercrosses is that the eyeless phenotype within these crosses could result from novel combinations of alleles from the two different surface populations, a phenomenon also called transgressive segregation. The presence of an eyeless phenotype resulting from between surface crosses suggests that cave-specific traits could result in areas where different surface phylogenetic lineages are brought together into secondary contact and hybridize. This might be the case for the high number of cave populations of *A. aquaticus* in Slovenia (besides the more apparent reason of high availability of subterranean habitats), for example, as at least three phylogenetically distinct surface lineages still live in proximity (Konec et al., 2015; Sworobowicz et al., 2015; Verovnik et al., 2005). Historical hydrogeological events might have caused secondary contacts that yielded hybrids with transgressive phenotypes, for example, loss of eyes, that were even more exapted for cave life than the normal surface ecomorph. These transgressive phenotypes could have resulted in faster and/or more frequent establishment of successful cave populations. If transgressive phenotypes are indeed a mechanism of cave population evolution, the hybrid history of the cave population should be detectable by inspecting genomes for alleles originating from different lineages. Whether or not this is a common mechanism involved in the evolution of cave characteristics in nature in *A. aquaticus* remains to be seen.

Transgressive segregation has been documented in multiple systems (reviewed in Mackay et al., 2021). In cichlids, two generalist species were intercrossed, and a phenotype present in specialized species (sand-sifting) was seen in the F_2 generation (Feller et al., 2020). Furthermore, it has been shown in *A. mexicanus* that introgression between surface and cave populations can be a mechanism for the evolution of cave-specific traits (Moran et al., 2022). Interestingly, the eye loss phenotype found in surface population intercrosses appears to have a different inheritance pattern than eye loss in the Planina Cave populations. In the Pivka and Rak Channels of the Planina Cave, a single gene appeared to be responsible for the eyeless phenotype. The

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extremely low frequency of eyeless individuals within the surface crosses is inconsistent for a single gene with full penetrance. Instead, multiple genes, incomplete penetrance, and/or variable expressivity could be responsible for this phenotype.

Also, there might be some element of developmental phenotypic plasticity as the two sides of the head can show different eye phenotypes; some individuals have complete eye loss with no pigmented eye spot and no ommatidia and then on the other side of the head there might be some sort of ommatidia and/or eye spot. Asymmetry of the two sides of the head is something that has been documented in cavefish regarding facial bone fragmentation and presence of neuromasts (Gross et al., 2016). So far, eyeless individuals (no ommatidia and no eye pigment on at least one side of the head) have only been seen in the orange eye Slovenian individuals crossed to the brown eye Romanian individuals and not the reciprocal cross (orange eye Romanian individuals to brown eye Slovenian individuals). However, one possible reason for this is that the reciprocal cross is currently in the F_2 generation, and the eyeless phenotype did not arise until the F_3 generation in the cross with orange eye Slovenian and brown eye Romanian parents.

Though we do not yet know how eye loss is inherited in the *A. a. infernus* population, crossing a surface population intercross individual with no ommatidia and no eye pigment on one side of the head and only ommatidia but no eye pigment on the other side of the head to an *A. a. infernus* individual did result in all individuals with both ommatidia and eye pigment indicating that if a single gene is responsible for eye loss in both populations and is inherited in a recessive manner, the same gene is likely not responsible for eye loss in both populations. However, it is unknown whether a single gene is responsible for eye loss in either the surface intercross individuals or *A. a. infernus*. Alternatively, the eyeless phenotype in the surface intercross individuals could not have a genetic basis and could be induced by some sort of environmental stress. Future studies will attempt to uncover whether there is, in fact, a genetic basis of the eye loss phenotype within the surface crosses, and if so, identify the genes and mutations responsible. If identification of the genetic basis of this phenotype is possible, it should elucidate whether standing variation and/or transgressive hybridization is responsible.

We also showed that the same region encodes the orange eye phenotype in the *A. a. infernus* and the Rak and Pivka Channels of Planina Cave populations. It is surprising that this "orange eye allele" appears to be present in multiple cave populations even though the phenotype that we associate with the variation (orange eyes) is masked within the cave populations due to lack of pigment apparently being epistatic to orange eyes. One could argue that mutations in multiple genes causing loss of pigment could be support for the overall degeneration of pigmentation pathways. However, it seems unlikely that degeneration of pigmentation pathways would happen in the same way with multiple cave populations being both unpigmented and having the hidden potential for being orange eyes. One possibility for the presence of orange eye alleles in these different cave populations is that if orange eye variation is present in the founding surface population, a newly evolved cave population first becomes orange if it is advantageous to have less or lighter pigment and the allele is already present in the population. Then over time, the population could evolve other mutations which result in complete lack of pigmentation. Or, it could not be a question of timing, but rather that the orange eye phenotype is a secondary consequence of what the allele does, and that the orange eye allele confers some sort of advantage in the cave environment. Pleiotropy of pigmentation phenotypes is not uncommon; phenotypes associated with pigmentation differences in insects include multiple behaviors, immunity, desiccation resistance and longevity (reviewed in Wittkopp & Beldade, 2009). Furthermore, in cavefish, loss of *oca2* was shown to be responsible for lack of pigmentation and increased tyrosine and catecholamine synthesis as well as sleep loss (Bilandžija et al., 2013, 2018; Klaassen et al., 2018; O'gorman et al., 2021). Also, in *A. mexicanus*, certain cave populations have yellow colored visceral adipose tissue which could have pleiotropic consequences (Riddle et al., 2020). Therefore, it is possible that the orange eye mutation in *A. aquaticus* has some pleiotropic function that is potentially adaptive, especially in the cave environment.

Complementation crosses support that the same gene is responsible for the orange eye phenotype in the populations examined including between the two surface populations, the Planina Cave and *A. a. infernus* cave populations, and between the *A. a. infernus* cave population and the Romanian surface population. Additional complementation crosses need to test all populations that hold the orange eye mutation. One caveat of the complementation cross is that noncomplementation could result if two different genes are responsible but are in the same pathway and interact with each other (second site noncomplementation). Examples of this have been shown in species including yeast and *Drosophila melanogaster* (reviewed in Hawley & Gilliland, 2006). However, since our noncomplementation results are supported by mapping results that show the genes are also in the same general location it seems likely that the same gene is responsible. For final confirmation that the same gene is responsible, the gene and mutations must be determined.

Interestingly, though this was not the focus of our study, crossing the Rak Channel of Planina Cave F_4

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individual to the *A. a. infernus* individual also showed unpigmented individuals and this phenotype was significantly associated with genotype at *disconnected* which marks lack of pigment in the Planina Cave populations. Therefore, it seems likely that the same region and gene is responsible for both orange eye and no pigment in the *A. a. infernus* population as compared to the Rak Channel of Planina Cave population.

In sum, we found that the same region, and perhaps the same gene, are responsible for the orange eye phenotype in the Rak Channel and Pivka Channels of Planina Cave populations, the *A. a. infernus* cave population, a surface Slovenian population and a surface Romanian population. This suggests that standing variation within the ancestral surface population could have been the source of the orange eye allele present within the cave populations. Additionally, the presence of the orange eye phenotype within cave populations which are geographically and ecologically distinct suggests that the orange eye phenotype might have some sort of pleiotropic function that might be advantageous in different environments. Finally, we found a common cave phenotype, eye loss, within a surface population or in surface population intercrosses suggesting that either standing variation and/or transgressive segregation could be a mechanism via which eye loss evolves in *A. aquaticus*.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All primers and sequences used in this report are present within the tables associated with this paper.

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