Behavioural Adaptation of Aquatic Cave Crustaceans: A Case Study in water lice *Asellus aquaticus*

Hajriz Berisha

Department of Systematic Zoology and Ecology
Biological Institute, Eötvös Loránd University
Budapest, Hungary

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Ph. D. Program of Environmental Biology, Doctoral School of Environmental Sciences at the Eötvös Loránd University, Budapest
Director: Tamás Turányi, DSc
Program Leader: Erika Tóth, DSc

Supervisor:
Prof. Gábor Herczeg
Department of Systematic Zoology and Ecology
Biological Institute
Eötvös Loránd University, Budapest

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INTRODUCTION AND OBJECTIVES

Cave ecosystems represent some of the most extreme and isolated environments on Earth, characterised by unique and challenging conditions. These environments are defined by perpetual darkness, stable temperatures, high humidity, and sometimes limited food resources, all of which significantly influence the organisms that inhabit them (Culver & Pipan, 2019; Howarth & Moldovan, 2018). In the absence of light, photosynthesis is not possible, meaning these ecosystems rely largely on external organic matter inputs or chemolithoautotrophic processes to sustain life (Simon et al., 2019). The study of these adaptations is crucial for understanding how life persists in such extreme environments and offers valuable insights into natural selection and evolutionary processes. Over the past century, research has increasingly focused on the unique adaptations of cave-dwelling organisms, often referred to as "troglobites," which include a variety of species that have evolved to live exclusively in subterranean habitats (Howarth & Moldovan, 2018). These adaptations are often extreme, involving morphological, physiological, and behavioural changes critical for survival in caves (Protas & Jeffery, 2012). One key behavioural adaptation observed in cave species is the modification of movement and foraging strategies to cope with the limited and sporadic availability of food. In the absence of light, many cave-dwelling species rely heavily on non-visual sensory modalities, such as chemoreception and mechanoreception, to navigate their environments and locate food (Soares & Niemiller, 2018). Additionally, the stable conditions in caves often lead to the evolution of slower metabolic rates and reduced reproductive cycles, which are adaptive strategies for conserving energy in environments where resources are scarce (Hervant et al., 1997).

The aquatic isopod crustacean *Asellus aquaticus*, serves as an excellent model for studying these evolutionary and behavioural adaptations. This species is widely distributed across Europe and North America and is highly eurytopic, meaning it can thrive in a wide range of environmental conditions, including both surface and subterranean habitats (Protas & Jeffery, 2012). The ability of *A. aquaticus* to colonise cave environments and develop troglomorphic traits, such as eye reduction and depigmentation, makes it a valuable subject for comparative studies of surface and cave populations (Pérez-Moreno et al., 2017; Balázs et al., 2021). Research on *A. aquaticus* has revealed significant behavioural differences between cave-adapted populations and their surface-dwelling counterparts. These differences are often linked to the distinct ecological pressures faced by cave populations, such as the absence of predators and the need to maximise foraging efficiency in a low-energy environment (Fišer et al., 2019). By comparing these populations, we can gain insights into natural selection processes and the role of behaviour in facilitating adaptation to extreme environments (Gibert & Deharveng, 2002).

This thesis aims to explore the behavioural adaptations of *Asellus aquaticus* in cave environments, focusing on three (movement activity, exploration, and risk-taking), from five main personality traits (including aggressiveness and sheltering). By examining these behaviours in both cave and surface populations, this research seeks to understand how *A. aquaticus* has adapted to the unique challenges of cave life and what these adaptations can reveal about broader evolutionary processes in extreme environments. The primary aim of this research was to examine the behavioural adaptations of *Asellus aquaticus* to the unique and challenging conditions of a cave environment. Specifically, the study focused on three key behavioural aspects:

1. **Movement Activity**: Assessing differences in movement patterns between cave and surface populations to understand how cave-dwelling individuals have adapted their locomotor behaviour for effective foraging and mate-seeking in a dark, resource-limited environment.
2. **Exploratory Behaviour**: Evaluating the extent to which cave populations differ from surface populations in their exploratory tendencies, with a focus on how energy conservation influences exploration in a nutrient-limited setting.
3. **Risk-Taking Behaviour**: Investigating whether cave-adapted populations exhibit different levels of risk aversion compared to surface populations, particularly in the absence of predators, to understand the evolutionary persistence of risk-related behaviours in isolated habitats.

STUDY ORGANISM AND SYSTEM

Asellus aquaticus is a widely distributed freshwater crustacean found across Europe and North America (Maltby, 1991; Turk et al., 1996; O'Callaghan et al., 2019). Known for its significant phenotypic plasticity, this species is capable of thriving in a variety of habitats, ranging from well-lit surface waters to the dark, isolated environments of caves. The primary focus of this study was the cave-dwelling population of *A. aquaticus* residing in the Molnár János Cave (MJC) in Budapest, Hungary. This cave, characterised by negligible predation, environmental heterogeneity, simplicity, stability and rich in food (endogenous bacterial mats), provides a unique ecological setting that has driven specific behavioural adaptations in its resident populations.

To provide a comprehensive comparison, the study also examined several surface populations of *A. aquaticus* from nearby aquatic environments, each offering different ecological challenges. The **Malom Lake (ML)** population, located near the entrance of the Molnár János Cave, experiences natural light, fluctuating temperatures, and the presence of predators such as fish. Interestingly, a group of colonists from this surface population has recently migrated into the cave, presenting a unique opportunity to study the early stages of cave adaptation.

In addition to these populations, the study included the **Dunakeszi Peat-moor (DM)** population, which inhabits a peat bog with variable light conditions and a complex community of both vertebrate and invertebrate predators. The **Csömör Stream (CS)** population was also studied, representing a freshwater stream environment with natural light cycles and a diverse array of predators. Lastly, the **Gotes Lake** population, another surface population with natural light cysles and exposure to various predators, was studied.

MATERIAL AND METHODS

Specimens of *Asellus aquaticus* were collected using different methods suited to the environmental conditions of each habitat. For surface populations, individuals were collected by hand-sorting through aquatic vegetation and sediment. In contrast, cave specimens were collected using a modified Sket bottle, a method adapted for the challenging conditions of cave environments, which required cave diving to reach the sampling sites. Upon collection, the specimens were transported to the aquaculture facilities at the Institute of Biology, Eötvös Loránd University. In the laboratory, individuals were housed in plastic Petri dishes (90 × 25 mm, diameter × height) with the bottoms roughened to facilitate normal locomotion. The Petri dishes were filled to half their height with water from the respective source habitats and regularly topped up to counteract evaporation. To ensure that the behaviour of the specimens in the experiments accurately reflected their natural responses, an acclimation period was implemented. Surface populations were acclimated to a 16-hour light and 8-hour dark cycle that mimicked their natural environment. Conversely, the cave population was kept in complete darkness to replicate the conditions of the Molnár János Cave. The acclimation period for all three studies lasted three to four days, during which the animals were left undisturbed to adapt to the laboratory conditions.

Experimental Setup

The behavioural experiments were conducted in custom-made recording chambers, each measuring 100 × 55 × 105 cm (length × width × height). These chambers were designed to allow precise control over the light conditions and to facilitate the accurate recording of animal behaviour. Each chamber was equipped with two types of lighting: overhead LEDs to simulate daylight conditions (colour temperature = 4500 K, colour rendering index >90) and infrared (IR) LEDs placed at the bottom for observations in darkness.

For each experiment, specific numbers of Petri dishes containing the animals were placed in the chambers, and the behaviour of the specimens was recorded using webcams (Logitech C920 FullHD) positioned to capture video footage under both visible and infrared light conditions. Video recordings were conducted using OBS Studio software.

Three key behavioural traits were assessed:

1. **Movement Activity:** Measured by tracking distance moved, time spent moving, and movement bouts under light and dark conditions.
2. **Exploratory Behaviour:** Assessed via maze experiments, focusing on the number of areas explored and dispersal speed.
3. **Risk-Taking Behaviour:** Evaluated by recording responses to a simulated predation threat, such as freezing or escaping.

Statistical Analysis

The data collected from the behavioural assays were analysed using a combination of linear mixed models (LMM) and generalised linear mixed models (GLMM). These models accounted for fixed effects such as population, sex, and light regime, as well as random effects, including individual variability and habituation effects. Principal Component Analysis (PCA) was also employed to reduce the dimensionality of the data and identify key behavioural patterns. All statistical analyses were performed using R version 4.1.2, with the lme4, lmerTest, and emmeans packages used for model fitting and post hoc comparisons. The results were interpreted with caution, considering potential limitations such as the artificial light conditions in the laboratory, which may not perfectly simulate natural cave environments.

RESULTS AND DISCUSSION

1. **The movement activity (in a familiar environment) is increased in the cave-adapted *Asellus aquaticus* population compared to surface conspecifics.**

   In the cave environment with negligible predation pressure, the ecological costs of movement activity are low, hence, movement activity can increase to maximise the benefits.

2. **The increase in movement activity (in a familiar environment) in the cave-adapted *Asellus aquaticus* population is higher in males than in females.**

   The benefit of movement activity is likely be higher for males due to the importance of their active mate searching behaviour females do not express.

3. **The explorativeness of the cave-adapted *Asellus aquaticus* population is decreased compared to surface conspecifics.**

   The benefits of explorativeness are decreased within the relatively homogenous and stable cave environment. Further, cave adaptations make successful dispersal out of the caves to the typical surface *Asellus aquaticus* highly unlikely.

4. **Surface morphs of *Asellus aquaticus* found in the cave (note that only active dispersal towards the cave is possible due to the strong water outflow) are more explorative than the average individual from their source surface population.**

   Only the boldest (including explorativeness) individuals are expected to disperse into a markedly different habitat, especially when the habitat boundaries are as narrow as in the surface – cave context.

5. **Surface-adapted *Asellus aquaticus* are more active (in a novel environment) during dark than during light.**

Dispersal/exploration might be less dangerous during night, when visually hunting predators are inactive. This behavioural strategy might be a preadaptation to the cave environment, allowing the successful colonisation of caves by *Asellus aquaticus*.

6. **In spite of the negligible predation pressure in the studied cave, cave-adapted *Asellus aquaticus* population showed no divergence in risk-taking behaviour from the surface populations.**

   The reason for this (lack of) pattern is yet unclear. Clarifying whether the pattern is true, and what might the reasons be behind warrants further investigations.

The research reveals that cave-dwelling populations exhibit higher movement activity compared to surface populations. This increase in activity, particularly among males, is likely an adaptive response to the negligible-predation, resource-scarce environment of the cave, where foraging and mate-finding require greater effort and energy expenditure (Fišer et al., 2019; Berisha et al., 2022). Moreover, the research notes that while surface populations are generally more explorative, cave-adapted populations demonstrate a reduction in exploratory behaviour (Horváth et al., 2023). The research also identifies that recent colonists from surface populations exhibit higher dispersal speeds and exploratory tendencies, supporting the hypothesis that bolder individuals are more likely to colonise new environments (Pérez-Moreno et al., 2017; Culver & Pipan, 2019). However, the study found negligible differences in risk-taking behaviour between cave and surface populations, raising questions about the evolutionary pressures in subterranean habitats (Soares & Niemiller, 2018). This finding suggests that certain behavioural traits, may be deeply ingrained and less susceptible to rapid evolutionary change. Overall, these results underscore the complex interplay between environmental conditions, evolutionary pressures, and behavioural adaptations in *Asellus aquaticus* (Gibert & Deharveng, 2002; Fišer et al., 2019).

Conclusions

The results from my doctoral studies suggest that the behavioral variation seen is the studied surface –cave *Asellus aquaticus* system is driven by different selective pressures. The negligible predation pressure and the seasonal and annual stability of the environment seems to be the key factors. However, patterns often differed between sexes, emphasizing that phenotypic variation in the wild is driven by natural selection operating *via* relative fitness, which is affected by environmental, sexual and fecundity selection in a habitat- and sex-specific way. This emphasises the importance of including both sexes in evolutionary population comparisons.

Variation in movement activity in a familiar environment followed my predictions, and could be explained by habitat-dependent predation pressure and sex-dependent benefits of the inreased activity under negligible predation risk. Other factors, such as food availability and population density, may also play a role, necessitating further study to fully understand *Asellus aquaticus* movement patterns.

Exploratory behaviour supports the idea that only the most explorative surface individuals colonize caves, but this drive decreases after adaptation. Later, as evolutionary adaptation to the unique cave habitat starts to take place over generations, explorativeness will provide no benefits within the homogenous and stable cave environments, while leaving the caves for cave-adapted individuals (e.g. having their eyes reduced and lost their pigments) is not really an option due to their highly likely inferiority in competitive or predatory situations. Therefore, I conclude that cave act as “dispersal traps”. Surface individuals exhibited more movement in dark, unfamiliar environments than in light, possibly due to reduced predation risk at night, a potential preadaptation for cave colonization. This could explain why *Asellus aquaticus* successfully colonized caves multiple times.

Predictions about risk-taking behaviour were not supported, suggesting that factors other than cave-surface differences may influence these variations. Finally, these findings are based on a single, specialized cave population and should not be generalized across all caves but seen as an example of how a species can adapt to a unique cave environment with low predation risk and abundant food.

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